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Antimicrobial effects of naturally occurring compounds against microorganisms relevant to food safety

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ANTIMICROBIAL EFFECTS OF NATURALLY OCCURRING COMPOUNDS AGAINST MICROORGANISMS RELEVANT TO FOOD SAFETY

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SUMMARY

Different types of antibiotics and chemical preservatives have been widely used in the agricultural food industry for various purposes including improvement of productivity and profits in modern farming practice, control of animal diseases and extension of shelf life of food products. However, due to increasing public concerns on the potential harmful effect posed by antibiotic residues in foods and the development of antibiotic resistance as a result of extensive use of chemical antibiotics in agriculture, the European Commission has banned the growth promoting antimicrobials in food animal production since Jan 2006. Research on the potential application of naturally occurring compounds in the food industry has recently gained increasing attention.

This study aim at evaluating the antimicrobial effects of naturally occurring compounds against pathogenic microorganisms relevant to food safety, public health, animal health and welfare. The overall objectives included the determination of the minimum inhibitory concentrations of the active compounds, investigation of the possible synergistic effects when combining different natural antimicrobial compounds and the investigation of antimicrobial modes of action of these active compounds.

Antibacterial activities of naturally-occurring compounds were evaluated against *Escherichia. coli* (*E. coli*), *Clostridium sporogenes* and *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). The most active compounds against these bacteria include 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, apple E polyphenols, green tea polyphenols, gallic acid, garlic powder, onion powder, onion oil, carvacrol, oregano oil, *trans*-cinnamaldehyde and cinnamon oil.

These compounds were subjected to antimicrobial mode of action studies. Significant cellular responses e.g. leakage of phosphate and reduction of intracellular ATP were observed. Monolayer study, a biophysical approach, suggested that the compounds interacted with cell membrane lipids, which was in agreement with the major findings of this study obtained using the biological approaches. A partial synergistic effect against

M. smegmatis MC^2155 was also observed when combining oregano oil and herbal extract Aw at sub-MIC concentrations.

PUBLICATIONS RELATED TO THIS THESIS

Wong, S. Y. Y., Grant, I. R., Friedman, M., Elliott, C. T. & Situ, C. (2008) Antibacterial activities of naturally occurring compounds against *Mycobacterium avium* subsp *paratuberculosis*. *Appl. Environ. Microbiol.,* **74**, 5986-5990.

ABBREVIATIONS

A_{260}	Absorbance reading at 260 nm
A_{π}	area per molecule at the indicated surface pressure π
ATP	Adenosine-5'-triphosphate
Aw	*Coptis chinensis Franch*
BA_{50}	bactericidal activities - a 50% decrease in the number of cfu
B. cereus	*Bacillus cereus*
$CaCl_2$	calcium chloride
cfu	colony forming unit
C. jejuni	*Campylobacter jejuni*
CL	cardiolipin
Cl. perfringens	*Clostridium perfringens*
Cl. sporogenes	*Clostridium sporogenes*
C_S	isothermal compressibility
C_S^{-1}	reciprocal isothermal compressibility / compressibility modulus
DMSO	Dimethyl sulfoxide $(CH_3)_2SO$
DNA	Deoxyribonucleic acid
DTAB	Dodecyltrimethylammonium bromide
DOPE	1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine
DOPG	1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt
DPPE	1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine
DPPG	1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	enterohaemorrhagic *E. coli*
FIC	fractional inhibitory concentration

FICI	fractional inhibitory concentration index
g	acceleration due to gravity
G state/phase	gas state/phase
GC/MS	gas chromatography-mass spectrometry
GRAS	generally recognised as safe
h	hour
HEYM	Herrold's Egg Yolk medium
HUS	haemolytic uraemic syndrome
LC state/phase	liquid-condensed state/phase
LE-LC state/phase	liquid-expanded/liquid-condensed state/phase
L. monocytogenes	*Listeria monocytogenes*
M	molar
Map	*Mycobacterium avium* subspecies *paratuberculosis*
min	minute
ml	millilitre
mM	millimolar (10^{-3} molar)
mN/m	milli Newton per metre
mV	millivolt
mg/ml	milligram per millilitre
MICs	minimum inhibitory concentrations
MRSA	methicillin-resistant *Staphylococcus aureus*
M. smegmatis MC^2155	*Mycobacterium smegmatis* MC^2155
ng/ml	nanogram per millilitre
nm	nanometre
NMR spectroscopy	Nuclear magnetic resonance spectroscopy
NPN	1-N-phenylnaphthylamine
OADC	Oleic Albumin Dextrose Catalase
OD_{600}	optical density at 600 nm
QToF LC/MS	Quadrupole Time-of-Flight mass spectrometry liquid chromatography / mass spectrometry
P. aeruginosa	*Pseudomonas aeruginosa*

PBS	phosphate buffered saline
rpm	revolution per minute
S. aureus	*Staphylococcus aureus*
SEM	standard error of the mean
S. typhimurium	*Salmonella. typhimurium*
Tris	Tris(hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultraviolet
% vol/vol	volume /volume percent
WHO	World Health Organization
% wt/vol	weight/volume percent
$Å^2$	square angstrom
°	degree
°C	degree Celcius
<	less than
>	greater than
≥	greater than or equal to
π	surface pressure
π-A	surface pressure-area
ψ	surface potential
$\Delta\psi$-A	surface potential-area
µg	microgram (10^{-6} gram)
µg/ml	microgram per millilitre
µl	microlitre (10^{-6} litre)
µl/ml	microlitre per millilitre
µM	micromolar (10^{-6} molar)
γ_0	surface tension of water
γ	surface tension in the presence of a lipid monolayer

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1 Literature review

1.1 Antibiotics

1.1.1 What are antibiotics?

The word "antibiotic" means agent "against life", these are substances that kill or inhibit the growth of microorganisms, including both bacteria and fungi (Walsh, 2003, Edwards, 1980). From time to time and in different places, the definition of antibiotics is varied; in general, the word antibiotics refers to natural products or semi-synthetic drug derivatives, which originally come from the antibiotic producing microorganisms. Moreover, it can also be referred to man-made synthetic chemicals (Walsh, 2003).

Antibiotic producing microorganisms include fungi and bacteria, the major group of antibiotic-producing bacteria is actinomycetes (Walsh, 2003). Antibiotics produced by microorganisms form the largest group of antimicrobial drugs (Edwards, 1980). Most of the antibiotics used in human medicine for treating infections in the past 60 years were natural products produced by microorganisms and semi-synthetic products derived from them (Walsh, 2003).

Antibiotics are mainly secondary metabolites (Edwards, 1980), they are produced when microorganisms enter the stationary phase. Microorganisms can regulate their gene expression to produce these antimicrobial metabolites when they are in competition for space, oxygen or nutrients, thus antibiotic-producing organisms have a selective advantage for growth since they can access essential growth elements from their dying neighbours (Walsh, 2003).

Antibiotics are assigned to various groups, depending on their chemical structures, elemental composition and physio-chemical properties (Korzybski et al., 1967). They generally have a low molecular weight, which means they are more easily absorbed by the cells of target; they can also inhibit the growth of microorganisms at low concentration (Lancini et al., 1995).

1.1.2 Mode of Action of Antibiotics

There are different cellular targets for antibiotics to exert their antimicrobial effects, the targets include cell wall biosynthesis, protein biosynthesis, DNA replication and repair, folate coenzyme biosynthesis (Walsh, 2003), cell membrane and function, and energy metabolism (Edwards, 1980). Some antibiotics act as antimetabolites, which are equivalent to competitive inhibitors, they have similar structures to normal metabolites, which can bind to the enzymes to alter their normal functions (Lancini et al., 1995).

There are two ways to describe the antimicrobial effect of antibiotics exerted on microorganisms, bactericidal or bacteriostatic. When sufficient damage is caused to one or more cell pathway or structure, the bactericidal response is triggered (Walsh, 2003). Bactericidal, refers to the killing of bacteria, such inhibition is permanent, meaning that bacteria lose their reproducibility even after the removal of the antimicrobial agents; while bacteriostatic means making the bacteria inactive, losing the ability to multiply temporarily in the presence of the antimicrobial agent but once the antimicrobial agents are removed, the bacteria can regain their reproducibility (Lancini et al., 1995). To treat an infection *in vivo*, it is not always necessary to administer bactericidal antibiotics. Bacteriostatic antibiotics can also be effective for controlling the spread of such pathogens, which are inactivated by the bacteriostatic drugs and then eliminated by the hosts' own immune system. Some antibiotics can be both bacteriostatic and bactericidal under different circumstances (Walsh, 2003).

Bacteria are classified into two main groups, Gram-positive and Gram-negative. Gram-positive bacteria refer to bacteria containing a high amount of peptidoglycan in the cell wall, and retain the crystal violet stain in a Gram stain test; while Gram-negative bacteria lack high amounts of peptidoglycan in the cell wall, instead they have an outer membrane, thus they cannot retain the crystal violet stain but take up the counterstain (safranin or fuchsin) and appearing red or pink. Some antibiotics are effective against Gram-negative bacteria while some against Gram-positive bacteria, as different antibiotics exert different mechanisms on different bacteria (Owens, 2004). Some antibiotics are effective against both, which are known as broad spectrum antibiotics.

However, there is no drug which can kill all kinds of infectious agents as therapeutics have their own specificities, targeting different microbes with different mechanisms (Walsh, 2003).

1.1.3 Antibiotics as Therapeutics

Since the discovery of the antibiotic penicillin, by Sir Alexander Fleming, antibiotics have been widely used in clinical medicine. The discovery of antibiotics was a corner-stone in the history of human medicine, as many lives have been saved since their application in therapeutics. It also led to the recognition that microbes are capable of producing different antimicrobial substances to inhibit the growth of their surrounding competitors, leading to intensive screening of microbial cultures in order to isolate new classes of antibiotics (Walsh, 2003).

Antibiotics can be specific for killing bacteria when they act on a target that is present in bacteria only but absent in humans and animals, for example the enzymes for cell wall biosynthesis and the folic acid biosynthetic pathway. Due to the structural differences in prokaryotic and eukaryotic DNA and protein synthesis machinery, selective inhibition is achievable (Walsh, 2003). Cell wall biosynthesis and protein biosynthesis of ribosomes have been the targets of most antibiotics, the possible reason is that these processes involve many enzymatic steps, which offer multiple opportunities for disruption and subsequently lead to inhibition (Walsh, 2003). Such selective actions only kill or inhibit the growth of target pathogens leaving the host unharmed (Edwards, 1980) and making antibiotics an ideal drug for treating microbial infection in humans and animals (Korzybski et al., 1967).

1.1.4 Antibiotics as animal feed additives

According to the Office of Health Economics (Office of Health Economics, 1969), the objective of animal husbandry is to produce large quantities of good quality meat or animal products in the most economically way possible. There are different approaches to achieve this objective; selective breeding is one good example in order to "proliferate the animals with desirable traits" as is providing good feed stuff for farm animals. The

combination of both approaches has brought huge improvements in the yield of food animals.

Besides applications in human therapeutics, antibiotics were also incorporated into feedstuffs as growth promoters in farm animals. In 1948, it was fortuitously discovered by Stokstad et al. (1949) that incorporation of antibiotics at very low concentrations (between 5 to 10 parts per million) as feed additives could significantly improve the growth of broiler chicken (Owens, 2004, Office of Health Economics, 1969). The exact mechanism of how antibiotics enhance the growth rate of poultry is still not fully known. However, it was found that antibiotics could improve weight gain, increase feed conversion efficiency, improve lean/fat ratio and improve animal welfare by improving the litter quality, meaning fewer bacteria are being shed in their droppings (Ross Tech, 1999). Antibacterial substances have since been extensively used in animal husbandry, for example in 1963, 40 percent by weight of all the antibacterial substances used in the United Kingdom was given to animals (Office of Health Economics, 1969).

Growth promotion of farm animals is important in order to meet the increasing demand of food products for the expanding worldwide population. Moreover, producing the greatest quantity of food from animals using the most economic way in the shortest possible time could generate better profit for livestock producers and help them maintain competitiveness while different food products are being imported from other countries, e.g. Brazil, where the cost of land, feed, labour and production costs are much cheaper (Owens, 2004).

1.1.5 Antibiotic Resistance

Though bacterial infections have been effectively controlled since the discovery and therapeutic application of antibiotics, this was, soon followed by the rapid development of antibiotic resistance (Gold and Moellering, 1996). Antibiotic resistance is a general phenomenon, which can be observed anytime and anywhere in the world (Lancini et al., 1995). To date, antibiotic resistance has become one of the most serious and challenging worldwide problems (White and McDermott, 2001). It has been suggested that the

widespread use of antibiotics has led to the continued emergence of antimicrobial resistance. One of the causes is due to the selection process of pre-existing naturally resistant organisms, the sensitive microbial cells within a population are eliminated, thus favouring the proliferation of the resistant cells (Edwards, 1980).

In fact, before the recognition of the existence of antibiotic producing organisms, resistance already existed. As microorganisms that produce antibiotics have self protection or autoimmunity mechanisms for protecting themselves from being harmed by the lethal chemicals produced by and within them, which means the antibiotic producers themselves can be resistant to antibiotics. Three main self protection mechanisms have been identified: the inactivation of an antibiotic, efflux of antibiotic and modification of the susceptible molecular target. The mechanisms also involve the exportation of antibiotics into the external medium in order to keep the intracellular concentration low. Some antibiotics are exported when they are still inactive, meaning that the inactive drug precursor is converted to the mature drug in the extracellular environment. Some organisms alter their cell wall, modify the enzyme peptidyltransferase, component of the protein synthesis machinery on the bacterial ribosomes, or produce desensitizing structural mutations in DNA replication enzymes (Walsh, 2003).

Development of antibiotic resistance in non-producers is in association with mutation, selection and transmission of such resistant genes either within the same species or interspecies. For microbes to survive in the presence of antibiotics released by the antibiotic producers in the natural environment, evolution occurs to devise resistance mechanisms and due to the large population and short generation time of bacteria, the development of mutant strain is facilitated (Walsh, 2003). In a population of microorganisms that is generally susceptible to antibiotics, there are likely to be some individual cells which survive from death or growth inhibition. Such individuals are called mutants, the frequency of such occurrence of mutants ranged between one mutant in every 10^7 to over 10^{10} sensitive cells (Lancini et al., 1995).

If this mutation of antibiotic resistance happens in one single bacterium in a patient receiving antibiotic treatment, in the *in vitro* cell cultural experiment, it would be found that the resistant cell proliferates and finally takes over the culture as their sensitive neighbours perish, thus the whole population would eventually be made up of just the antibiotic resistant cells (Lancini et al., 1995, Walsh, 2003). The more wide spread the use of antibiotics, the more this enhances the chance of this natural selection. This is believed to be the mechanism of how antibiotics enhance the selection of antibiotic resistant bacteria and promote their development (Edwards, 1980, Walsh, 2003).

In fact, such mutation is actually a normal and natural phenomenon which occurs both in the laboratory and in the environment. Another natural phenomenon could follow, which is known as back mutation, which means that the antibiotic resistant mutants would revert back to a sensitive cell in the absence of an antibiotic, thus in the long term the population would revert back to sensitivity (Lancini et al., 1995).

1.1.6 Transfer of antibiotic resistance to other microorganisms

Besides mutation and selection, another cause of antibiotic resistance is the transfer of resistant genes among bacteria. The resistant genes contained either in chromosomal DNA or plasmid DNA could be transferred from one bacterium to another, by conjugation, transduction or transformation (Edwards, 1980, Lancini et al., 1995). The resistance mechanisms found in resistant strains appear to be acquired from antibiotic producing bacteria (Walsh, 2003). Webb and Davies (1993) reported the presence of genetic materials, including the antibiotic resistant genes for self protection from antibiotic producers, in antibiotic therapeutics for both humans and animals, which may pose a risk of transferring the antibiotic resistant genes to pathogenic bacteria in animal and human guts after administration. It has been reported that multiple drug resistant plasmids have been transferred between bacteria from different genus and species (Kruse and Sørum, 1994), as the transmission does not necessarily occurr within the same species, which means it might greatly increase the chances of possible transmission. In some other cases, if one resistant individual is present in a population of microorganisms and able to transfer the resistant genes to the entire population, then the

microbial population does not need to go through the natural selection process but acquires the resistance; such phenomenon is known as "transferable resistance" (Lancini et al., 1995), which may significantly increase the occurrence of resistance.

In order to tackle the problem of resistance, two approaches have been taken; firstly, research to discover new classes of drugs, secondly chemical modifications of existing drugs. However, the development of new drugs might not be able to keep pace with the ability of pathogens to develop resistance (Gold and Moellering, 1996).

1.1.7 EU-wide ban on incorporating antibiotics in animal husbandry

There are three possibilities as a result of the use of antibiotics in farm animals that could pose risks to human health (Piddock, 1996). Firstly, the use of antibiotics might promote the selection of resistant bacteria in farm animals; if such bacteria are pathogenic to human and if they happen to contaminate the foodstuff during slaughtering or food preparation, infection caused by such resistant bacteria would be difficult to treat. Secondly, the use of antibiotics in farm animals could promote the selection of resistant bacteria that are non-pathogenic to humans, those bacteria might again contaminate the foodstuff and enter the human gastrointestinal tract and transfer their resistant genes to the microflora in the human gut. Thirdly, the antibiotics might remain as residues in the farm animal tissues, after consumption of those meat products, selection of resistant bacteria in the human gastrointestinal tract would be allowed.

Resistant strains of bacteria from different genuses and species are isolated from humans as well as different animals in different parts of the world (Griggs et al., 1994, Sandvang et al., 1997, Blanco et al., 1996, Blanco et al., 1997, Everett et al., 1996). For instance, *Salmonellas* isolated from poultry in the UK (Wray et al., 1992), *E. coli* isolated from humans and animals in India (Singh et al., 1992), and *Enterococci* isolated from turkey in the USA (Welton et al., 1998). Due to the ever-increasing public concern on issues of antibiotic resistance, in 2001, it was proposed to ban the use of all kinds of antibiotics as animal growth promoters in farm animal production in Europe. The new legislation eventually came into effect in January 2006 (Official Journal of the European Union,

2003). Some researchers did not agree with the ban of using antibiotics as growth promoters in animal productions, for example, Phillips et al. (2004) concluded that the precautionary principle was not scientific, however, it did not change the implementation of the ban.

1.1.8 Consequences of banning antibiotics in animal husbandry

Since the proposition of such a ban, livestock producers and researchers have been actively looking for alternative feed additives in order to prevent and control diseases in farm animals and so as to maintain good performances in animal production. Different feed additives include essential oils (Shanmugavelu et al., 2006, Owens, 2004, Mitsch et al., 2004, Lee et al., 2003, Schöne et al., 2006), secondary plant metabolites (Shanmugavelu et al., 2006), plant extracts (Jamroz et al., 2005), organic acids, enzymes, probiotics, prebiotics, symbiotic, yeast extract (Owens, 2004), mushrooms, herb polysaccharides (Guo et al., 2004c, Guo et al., 2004b), Chinese herbal medicine (Guo et al., 2004a) and other dried herbs (Bampidis et al., 2005) etc. have been extensively studied to determine their potential to replace and/or reduce the use of antibiotics in farm animal production.

1.2 The microorganisms

1.2.1 *Clostridium*

Clostridium is a Gram-positive organism, most species are obligate anaerobes and are capable of forming spores under adverse growth conditions, e.g. when nutrients are depleted. For most species, the optimum growth temperature is between 30-37°C (Cato et al., 1986), which is very close to the basal body temperature of mammals. When spores are formed, they are oval or spherical and usually distend the cells. Most strains are straight or slightly curved rods of varying length and width (Cato et al., 1986). Some species are human or animal pathogens or pathogenic for both. They are ubiquitous, commonly found in soil, sewage, decaying vegetation, animal and plant products, in the intestines of humans, other vertebrates and in wounds or soft tissue infections of humans and other animals (Cato et al., 1986).

1.2.2 *Clostridium perfringens*

Clostridium perfringens is a member of genus *Clostridium*, producing a variety of virulence factors. They produce a number of soluble substances that exert a variety of toxic effects *in vitro*, *in vivo*, or both. They have been divided into five types (A, B, C, D and E) on the basis of the production of the major lethal toxins, alpha, beta, epsilon and iota (Cato et al., 1986).

1.2.3 Disease in farm animals and humans cause by *Clostridium perfringens*

Some species are pathogenic for humans, or animals, or both. *Cl. perfringens* food poisoning in humans is produced by type A strains, which may produce heat-sensitive spores or heat-resistant spores (Cato et al., 1986). Spores that survive cooking, during slow cooling and unrefrigerated storage may germinate and proliferate to high counts in food products, usually warm meats or meat products; when ingested, the vegetative cells sporulate in the gut and release toxin (HPA, 2008b). Enterotoxin is a product of the sporulation process and can cause fluid accumulation in the small intestine of laboratory animals. Feeding of purified enterotoxin to volunteers reproduces the food poisoning syndrome (Cato et al., 1986).

In addition to causing food poisoning in humans, the toxin also causes muscle death of myonecrosis in case of *Cl. perfringens* gas gangrene occurs, a rare but very severe form of tissue death in humans and other animals following trauma or abortion. According to Smith (Smith, 1979), the mechanism of action of these factors usually falls into one of the following three groups. First, some of the virulence factors, such as the alpha toxin, which is phospholipase C produced by all five types of *Cl. perfringens* and the kappa toxin, which is a collagenase and are enzymes, could hydrolyze substances, e.g. membrane lecithin that are essential to the integrity of membranes or other body structures (Cato et al., 1986). Secondly, other virulence factors, such as the beta, epsilon and iota toxins, act primarily on the vascular endothelium, causing increased capillary permeability especially in the brain, thus increasing the permeability of other virulence factors entering the blood brain barrier. Thirdly, other factors, such as the delta and theta

toxins, which are essentially haemolysins, could cause lysis of blood cells. *Cl. perfringens* is responsible for 80-95% of cases (HPA, 2008a).

Rood et al. (1978) reported isolation from pig faeces of a number of strains that were resistant to tetracycline, erythromycin, lincomycin and clindamycin; the degree of resistance appeared to correlate with the use of antimicrobial-containing animal feed.

1.2.4 Sources and transmissions of *Clostridium perfringens*

Cl. perfringens is the species of *Clostridium* most commonly isolated from most of the polymicrobial infections in humans (Cato et al., 1986). *Cl. perfringens* has been isolated from the intestinal contents of virtually every animal investigated. Humans frequently carry *Cl. perfringens* as part of the normal endogenous flora, e.g. in normal oral flora and from urine but the main site of carriage is still the gastrointestinal tract. *Cl. perfringens* is more widely spread in nature than any other pathogenic microorganisms. Sources of *Cl. perfringens* include soil and marine samples worldwide, clothing, raw milk, cheese and semi-preserved meat products.

1.2.5 *Clostridium sporogenes*

Clostridium sporogenes are also a member of the genus *Clostridium*, they are Gram-positive straight rods. Spores are oval, subterminal; sporulation occurs readily on most media. Following sporulation, the vegetative material may disintegrate rapidly to leave only free spores. Surface colonies on blood agar are 2-6 mm in diameter, irregularly circular, posses a coarse rhizoid edge, have a raised yellowish gray centre and a flattened periphery composed of entangled filaments, which could also be referred as "Medusa head" colonies, are opaque, possess a matt surface and are usually β-hemolytic (Cato et al., 1986). Such characteristics remain the same when cultured on brain heart infusion agar.

1.2.6 Sources, role in infections and food spoilage of *Clostridium sporogenes*

This species have been isolated from a very broad range of sources, including soils throughout the world, marine and fresh water lake sediment, preserved meat and dairy

products, snake venom, faeces of sheep and dogs, human infant and adult faeces; from infection of domestic animals; from human infection including bacteraemia, infective endocarditis, central nervous system and pleuropulmonary infections, abscesses, war wounds and other pyogenic infections (Cato et al., 1986). The toxicity of cultural supernatants of *Cl. sporogenes* was tested on mice, which was negative. Although *Cl. sporogenes* is isolated from infections, these infections are usually polymicrobial. This species is not yet known to have any role as a pathogen in such infections. The highly proteolytic nature of *Cl. sporogenes* is thought possibly to act as an adjuvant and promote invasiveness of other bacteria in various mixed infections of animals and humans, which resembles a synergistic effect (Cato et al., 1986).

1.2.7 *Escherichia coli*

Escherichia coli (*E. coli*) are Gram-negative straight rods, 1.1-1.5 μm x 2.0-6.0 μm, occurring singly or in pairs. They are facultative anaerobes, having both a respiratory and a fermentative type of metabolism. Their optimum growing temperature is 37°C, which is the normal body temperature of mammals (Ørskov, 1986). They are commonly found in the gut, mainly the lower part of the intestine of humans and other warm-blooded animals. Most strains of *E. coli* are harmless. However, some strains such as enterohaemorrhagic *E. coli* (EHEC) produce enterotoxins, they are known as enterotoxigenic strains, which can cause severe foodborne disease (WHO, 2005).

1.2.8 *Escherichia coli* K12

E. coli K12 is a non pathogenic strain of *E. coli* that can be used as a surrogate of the pathogenic strain for research when a containment level 3 laboratory is not available.

1.2.9 *Escherichia coli* O157:H7

The toxin produced by EHEC is known as verotoxins or Shiga-like toxins due to their similarity to the toxins produced by *Shigella dysenteriae*. The optimum growth temperature for EHEC is 37°C but it is also capable of growing in temperatures ranging from 7°C to 50°C. Some EHEC can grow in acidic foods and in foods with a minimum water activity. It could be completely inactivated by thorough cooking of foods until all

parts reach a temperature of 70°C or higher. *E. coli* O157:H7 is the most important EHEC serotype in relation to public health, most available information is related to serotype O157:H7 since it is easily differentiated biochemically from other *E. coli* strains. However, other serotypes have frequently been involved in sporadic cases and outbreaks as well (WHO, 2005).

EHEC infections, causing internal bleeding, are important foodborne diseases, which have emerged over recent decades. Although their incidence is relatively low, their severity and sometimes fatal health consequences, particularly among infants, children and the elderly, make them among the most serious foodborne infections (WHO, 2007).

1.2.10 Disease in farm animals and humans cause by *Escherichia coli* O157:H7

The significance of EHEC as a public health problem was recognized in 1982, following an outbreak in the United States of America. Clinical symptoms include abdominal cramps and diarrhoea that may in some cases progress to bloody diarrhoea, also known as haemorrhagic colitis. Fever and vomiting may also occur. Most patients recover within 10 days, but in a small proportion of patients, particularly young children and the elderly, the infection may lead to a life-threatening disease, such as haemolytic uraemic syndrome (HUS). HUS is characterized by acute renal failure, haemolytic anaemia and thrombocytopenia. It is estimated that up to 10% of patients with EHEC infection may develop HUS, with a case-fatality rate ranging from 3% to 5%. Sixty-three to 85% of infections are a result of ingestion of contaminated food (WHO, 2005).

1.2.11 Sources and transmissions of *Escherichia coli* O157:H7

According to the World Health Organization (WHO, 2005), the reservoir of this pathogen appears to be mainly cattle and other ruminants such as camels. EHEC is transmitted to humans primarily through ingestion of contaminated food products, such as raw or undercooked ground meat products including dried cured salami, raw milk and other dairy products. An increasing number of outbreaks have been associated with the consumption of fresh fruits and vegetables and their products e.g. unpasteurized fresh-pressed apple cider, sprouts, lettuce, coleslaw and salad, whereby contamination may

occur due to contact with faeces from domestic or wild animals at some stage during cultivation or handling. Cross-contamination might also occur during food preparation when kitchen utensils and work surface are contaminated.

EHEC has been found to survive for months in manure and water-trough sediments. Waterborne transmission has been reported, from both contaminated drinking-water and from recreational waters. Person-to-person contact is also an important mode of transmission through the oral-faecal route. It has been reported that a carrier showing no clinical sign of the disease are capable of infecting others. Visiting farms and other venues where the general public might come into direct contact with farm animals has also been identified as an important risk factor for EHEC infection as well (WHO, 2005).

1.2.12 *Mycobacterium*

Mycobacteria are aerobic, nonmotile, slow-growing, rod-shaped bacteria, either slightly curved or straight and 0.2-0.6 x 1.0-10 μm in size. They do not form endospores and not readily stainable by Gram's staining method but usually considered as Gram-positive. They are characteristically acid-fast due to the waxy materials presence in their complex cell walls, which is particularly important for recognizing this genus. After staining they resist decolourization with acidified alcohol. *Mycobacterium* has traditionally been considered to be rather separate, and is thus usually treated as a family, the *Mycobacteriaceae*, the genus is the only one in its family. However, they are also closely related to the genera *Corynebacterium* and *Nocardia*. Genus includes obligate parasites, saprophytes and intermediate forms (Wayne and Kubica, 1986).

The hydrophobic nature of *Mycobacteria* is associated with their high lipid content, which may contribute part of the reason of their slow growth (Harshey and Ramakrishnan, 1977). Most species adapt readily to grow on very simple substrates using ammonia and amino acids as nitrogen sources and glycerol as carbon source in the presence of mineral salts but some fastidious organisms require supplements such as haemin, mycobactins or other iron transport compounds for growth *in vitro*, for example *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) (Wayne and Kubica, 1986).

1.2.13 *Mycobacterium avium* subspecies *paratuberculosis*

Mycobacterium avium subspecies *paratuberculosis* (*Map*) was once known as *Mycobacterium paratuberculosis*, another common name was Johne's bacillus. They are plump rods, 1-2 μm in length (Wayne and Kubica, 1986). This organism is very difficult to cultivate. All *Mycobacteria* produce mycobactins, which are essential for mycobacterial growth, except *Map*. When growing *in vitro*, mycobactin J is required, such requirement could be useful in distinguishing this strain from the others. Colonies are usually visible after 6 weeks of incubation at optimum growth temperature, 37°C, from dilute inoculum. However, when cultivating *Map* from other samples, e.g. clinical, environmental and food samples, it would require three to four months (Ortegon, 2007) to produce visible colonies.

1.2.14 Disease in farm Animals and humans cause by *Mycobacterium avium* subspecies *paratuberculosis*

Map is the causative agent of Johne's disease, which is also known as Paratuberculosis in wild and domestic ruminants, especially dairy cattle and other ruminants (Clarke, 1997, Wells and Wagner, 2000, Saxegaard, 1985, Hirst et al., 2004, Roussel et al., 2005, McKenna et al., 2006). *Map* is apparently an obligate parasite in nature (Wayne and Kubica, 1986). *Map* may also relate to Crohn's disease and diabetes in humans (Naser et al., 2004, Hermon-Taylor et al., 1998, Scanu et al., 2007, Uzoigwe et al., 2007).

Johne's disease is a chronic granulomatous enteropathy (Berger et al., 2007), clinical signs include chronic diarrhoea, progressive weight loss, decreased milk production and infertility (Cocito et al., 1994). There is currently no approved drug or treatments for Johne's disease (Parrish et al., 2004). Once farm animals are diagnosed with Johne's disease, they are culled early (Rowe and Grant, 2006). This leads to economic loss of livestock and dairy producers due to reduced productivity, animal wasting and mortalities in severe cases (Berger et al., 2007, Rowe and Grant, 2006).

1.2.15 Sources and transmissions of *mycobacterium avium* subspecies *paratuberculosis*

Johne's disease occurs worldwide (Clarke, 1997). Transmission of *Map* is through faecal-oral route between animals (Parrish et al., 2004). Calves under six months old are the most susceptible to infection among different age groups (Ortegon, 2007) when their immune system is not fully developed. Before the onset of the clinical symptoms, there is a latent period that could last for at least two years. During the latent period the carrier animals could infect surrounding animals and the farm environment through the shedding of the *Map* into their faeces (Clarke, 1997). A farmer only realises that one of their animals is infected with *Map* when clinical signs are observed. At that stage the farm and other animals within the herd will have been already exposed to *Map* infection.

Milk could be contaminated with *Map* as it is systemic (Sweeney et al., 1992) or by faeces from infected animals during milking (Grant et al., 2002), which could be transmitted to calves and humans. Another possible route of transmission of *Map* from cattle to humans is via contaminated water (Whan et al., 2001).

1.2.16 Resistance to antibiotics, pasteurization and other physical treatments of *mycobacterium avium* subspecies *paratuberculosis*

Map is resistant to a variety of environmental degradations, it survives in stagnant water, manure, deep soil and freezing temperature minus 14°C for up to one year and so as many disinfectants (Ortegon, 2007). Studies have shown that *Map* can survive pasteurisation (Grant et al., 1996, Grant et al., 1998, Grant et al., 1999, Grant et al., 2002, Grant et al., 2005), which might pose risks to human health. Attempts to inactivate *Map* include the use of UV irradiation (Altic et al., 2007), pulsed electric fields (Rowan et al., 2001) and hydrostatic pressure (López-Pedemonte et al., 2006).

Mycobacteria are relatively resistant to most of the broad spectrum antibiotics, with the notable exceptions of streptomycin and rifampicin (Wayne and Kubica, 1986). The antimicrobial resistance of *Mycobaceria* is intrinsical (Nguyen and Thompson, 2006). As described by Nguyen and Thompson (2006), the mechanisms include a passive

exclusion of antimicrobials by their complex and impermeable cell wall and lacking of porin channels, an efflux pumps to eliminate antibiotics, producing enzymes to modify or degrade antibiotics, and modifying their own enzyme thus the antibiotic could no longer bind to it, etc.

1.2.17 *Mycobacterium smegmatis* MC^2155

Mycobacterium smegmatis (*M. smegmatis*) MC^2155 is considered as a fast-growing *Mycobacterium*. They can be isolated from the skin of humans. Due to its relative fast grow rate, *M. smegmatis* MC^2155 is applied in phage assay, in order to help to enumerate the number of viable cells of slow growing pathogenic *Mycobacterium*, e.g. *Mycobacterium tuberculosis* in clinical samples and *Map* in food samples. Apart from these, *M. smegmatis* MC^2155 can be used to serve as surrogate for the slow growing pathogenic *Mycobacterium* in preliminary antimicrobial screenings.

1.3 Naturally Occurring Compounds

As suggested by the name, naturally occurring compounds refer to any substances obtained naturally from either plant or animal origins. It also refers to the crude extracts of plants and animals or their purified and isolated constituents.

1.3.1 Major groups of plant-derived naturally occurring compounds as antimicrobials

According to Cowan (1999), antimicrobial compounds from plant origins could be classified as the following major groups, phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides and polyacetylenes; and the group phenolics, could be further classified into different sub-groups, they are simple phenols, phenolic acids, quinones, flavonoids, including flavones and flavonols, which are subgroups of flavonids, tannins and courmarins.

1.3.2 Phenolic compounds

The simplest form of phenol consists of a single substituted phenolic ring, cinnamic and caffeic acid are the common representatives of phenolic acids (Cowan, 1999). Carvacrol,

the main constituent isolated from oregano oil is another example of phenolic compound. Polyphenols from green tea are well known of their antioxidant properties and possible beneficial effect to health of humans.

1.3.3 Essential oils

The fragrance of plants is contributed to by the essential oil fraction, which is secondary metabolite. They are highly enriched in compounds based on an isoprene structure, known as terpenes, or terpenoids when additional elements are contained, usually oxygen (Cowan, 1999). It has been suggested that the antimicrobial activity of herbs and spices is mainly contributed from the essential oil fraction (Deans and Ritchie, 1987).

1.3.4 Sources of plant-derived naturally occurring compounds

Naturally occurring plant compounds refer to secondary plant metabolites and are obtained naturally from different parts of plant materials, common herbs, spices and teas, including, flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots by steam distillation, expression, fermentation or extraction (Prabuseenivasan et al., 2006).

1.3.5 Naturally occurring compounds from animal origins as antimicrobials

Animals can produce antimicrobial peptides, which are important as they function in the first-line of the host defence against different pathogens including bacteria, fungi and viruses (Sahl et al., 2005). These peptides could be found in different animals, e.g. skins of amphibians, they are secreted from the subcutaneous glands; they could be obtained directly from the amphibians' skin or synthesized using the molecular cloning technology. Apart from their natural antimicrobial properties, amphibian peptides have also been studied for their anti-cancerous properties.

1.3.6 Use of naturally occurring compounds in traditional folk medicines

There are thousands of different plant species which have been used in folk medicines due to their healing powers since ancient times worldwide (Cowan, 1999). After the discovery and application of antibiotics in the 1920s, the use of plant derivatives as antimicrobials was nearly abandoned. The emergence of antibiotic resistance has led to

the realisation that the life span and effectiveness of any antibiotics is limited. Applications of natural remedies as alternative medical treatments have become popular since late 1990s (Cowan, 1999). Many naturally occurring compounds, such as essential oils and phenolic compounds (Friedman et al., 2006b, Friedman et al., 2004a, Friedman et al., 2002, Friedman et al., 2003, Friedman et al., 2004b) have been evaluated extensively for antimicrobial activity and chemically characterized.

1.3.7 Reasons for researching natural occurring compounds

Food-borne infections, which may affect any individuals, have been a worldwide health concern (Brandi et al., 2006). Besides infections, concern of food safety in relation to antimicrobial growth promoters (AGPs), hormones, food additives and food preservatives have also been raised recently (Brewer et al., 1994). In the past, chemical preservatives had been used excessively in food manufacturing, however, the safety of such synthetic substances remains a question, due to their possible potential toxicity, carcinogenicity and teratogenicity, food manufacturers have been pressurised to minimise or even omit their use in food processing completely, in order to maintain the wholesomeness and quality of food products (Tassou and Nychas, 1995). The need for natural, safe and effective antimicrobial preservatives as an alternative of chemical preservatives is increasing (Kwon et al., 2003). The use of herbs and spices, which are naturally occurring compounds from plant origins, as alternative antimicrobials have been recently renewed again (Brewer et al., 1994). Numerous studies on the antimicrobial activities of herbs and spices against foodborne pathogens have been carried out either in food matrices, buffer or growth medium (Juglal et al., 2002, Smith-Palmer et al., 1998).

Many naturally occurring compounds, especially from herbs and spices and their extracts have already shown prominent antimicrobial activities (Tassou and Nychas, 1995). Some tea extracts were found to be more effective on several bacteria than currently used antibiotics at similar concentrations (Friedman et al., 2006b). Other compounds such as phenols, essential oils and some of their purified and isolated components were also effective against some food borne pathogens (Friedman et al.,

2002, Friedman et al., 2003, Friedman et al., 2004b) and some of them may have an effect on antibiotic-resistant pathogens (Friedman et al., 2004a). Tea polyphenols and their derivatives are thought to be important to maintain human gastrointestinal health (Lee et al., 2006).

They are ideal for study as most of them are considered to be 'Generally Recognised As Safe' (Kabara, 1991). Moreover, the naturally occurring compounds from plant origins are also effective as they involve in self dense of the plants against microbial invasion. Some naturally occurring compounds have a wide spectrum activities against different microbes, examples of these compounds are thymol from oregano, cinnamic aldehyde from cinnamon and eugenol from cloves (Beuchat and Golden, 1989).

1.3.8 Use of naturally occurring compounds in animal husbandry

1.3.8.1 Control the growth of pathogens and prevent diseases

Naturally occurring compounds can be incorporated into animal feedstuffs to help to minimize microbial infection and inhibit those animal microflora that are pathogenic to humans, e.g. to decrease the shedding of *E. coli* O157:H7 in faeces in cattle (Wells et al., 2005). *E. coli* O157:H7 carried in the food animal might be passed to humans directly through consumption, or passed into the environment and then to humans again through faecal contamination of drinking water and irrigation of crops (WHO, 2005).

Mitsch *et al.* (2004) studied the effect of two different blends of essential oil components on the proliferation of *Cl. perfringens* in the intestines of broiler chickens. It was found that the compounds could help to control the growth of this pathogen in the gut, and they concluded that they may be used to help preventing necrotic enteritis and other problems associated with *Cl. perfringens* like food poisoning in humans.

1.3.8.2 Enhance growth performances

Guo *et al.* (2004c) demonstrated that incorporating a mixture of mushroom and herb polysaccharides into broiler's feed enhanced growth performances, both body weight

gain and the feed conversion efficiency had increased, such effects were similar to broiler group fed with antibiotic growth promoter virginiamycin. In another study, Bampidis *et al.* (2005) found that dietary dried oregano leaves increased feed conversion efficiency in early maturing turkeys, despite the feed intake being decreased.

Jamroz *et al.* (2005) studied the incorporation of capsaicin, cinnamaldehyde and carvacrol in chicken diets based on maize or wheat and barley. Though the body weight was not enhanced, feed conversion index was significantly improved and breast muscle was enhanced. The possible reason of such enhancement might be due to the better utilisation of nutrients of feed as was indicated by a significant reduction of intestinal flora, *E. coli* and *Cl perfringens* found. This could also be the result of the antioxidant activity of the naturally occurring compounds which might protect the intestinal villi thus positively affect the absorption of digested nutrients.

1.3.9 Research into naturally occurring compounds as food preservatives

There are two approaches which can be used to ensure food safety and to extend shelf life of food products, to inhibit the growth of food-borne pathogens in food or to delay the growth of food spoilage microbes thus to delay the onset of food spoilage (Tassou and Nychas, 1995). Extensive studies have been carried out to examine the antimicrobial activities of different naturally occurring substances e.g. plant extracts and essential oils; and as well as their potential use in food preservation.

Fisher and Phillips (2006) studied the effect of lemon, orange and bergamot essential oils and their components on the survival of a number of common foodborne pathogens *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus in vitro* in food models cabbage leaves and chicken skin. They also found that Gram-positive bacteria were more susceptible to the antimicrobial test than Gram-negative bacteria in general.

These studies suggested that application of naturally occurring compounds could help to improve food safety, food quality, extend shelf life and for safer and more efficient food animals production.

1.3.10 Different assay methods to determine the antimicrobial activities of naturally occurring compounds

Different approaches are used to determine the antimicrobial activities of naturally occurring compounds against different types of bacteria, e.g. micro/macro-broth dilution method (Galindo-Cuspinera et al., 2003), agar-disc diffusion method (Galindo-Cuspinera et al., 2003), agar-well diffusion method (Magwa et al., 2006), using matrices without any carbon sources nor other nutrients, e.g. physiological saline (0.85% NaCl) (Kwon et al., 2003) and buffer (Friedman et al., 2002, Friedman et al., 2003, Friedman et al., 2006b), and so as food matrices, such as milk, cheese (Abbasifar et al., 2009), salad (Cobo Molinos et al., 2009), vegetable broth (Valero and Francés, 2006), cooked ground beef (Juneja et al., 2006) and apple juice (Friedman et al., 2004b) etc.

Different approaches might result in different antimicrobial activity being observed and indicated as minimum inhibitory concentrations (MICs), for the same compounds against the same bacteria. It is because the use of different assays might affect the availability of the test compounds to the bacterial cells, e.g. when testing compounds that do not have an ability to diffuse uniformly in solid medium, agar-disc and agar-well diffusion method should be avoided, otherwise the true potency of the compounds could not be revealed, as the test compounds available to the bacterial cells would be limited due to the poor diffusibility.

Different matrices might affect the interaction between the compound molecules and the bacterial cells, e.g. the complex nutrients contained in growth media and food models might make the test compounds more or less available to the test organisms. Examples are testing oil compounds in food models containing high fat content, the test compounds might partition in the fat molecules of the food and become less available to the test organisms, while testing oil compounds in food models with high acidity, the

hydrophobicity of the test compounds might be enhanced and become more available to the test organisms.

Moreover, the number and phase of growing bacteria used in the test could also be a factor affecting the MICs. The definition of MICs, whether complete inhibition, or partial, e.g. 50% inhibition, could be different in separate studies and whether the test aims to determine the bacteriostatic or bactericidal effect of the test compounds could be different, too, which makes comparison between different studies difficult (Friedman et al., 2002). Some researchers suggested standardisation of those tests is necessary to allow easy and direct comparison; however, it is difficult to apply such standardisation as there are different objectives for researches, different microorganisms have different nutritional requirements and whether the microorganisms are fastidious should be taken into account for the selection of appropriate antimicrobial assay.

1.3.11 Importance of determination of minimum inhibitory concentrations MICs

As most of the essential oils have their own distinct organoleptic characteristics, consumers' appreciation of these characteristics will be subjective. Determination of minimum inhibitory concentrations (MICs) is therefore important, in order minimum amount of compounds could be applied in food products to achieve inhibition of growth of microbes, which would result in least impact on flavour that would not exceed the threshold acceptable to consumers (Nazer et al., 2005).

1.3.12 Determination of possible mechanisms of action

A number of studies have been carried out determining the possible modes of action of some naturally occurring compounds on different bacteria using different approaches. The solubility of essential oils in the phospholipid bilayer of biological membranes plays an important role in their antimicrobial activities (Knobloch et al., 1989). Essential oils cause damage to biological membranes possibly due to their lipophilic properties and some specific functional groups were additionally effective (Knobloch et al., 1989). Terpenoids have been found to interfere the enzymatic reactions of energy metabolism in microbes (Knobloch et al., 1989).

In order to determine the mode of action and to study how the naturally occurring compounds interact with the microorganisms, different parameters can be used and measured. For example, study of cell homeostasis, including ATP synthesis (Gill and Holley, 2004) and intracellular pH (Ultee et al., 1999); whole cell autolysis (Gustafson et al., 1998); direct observation of cell morphology subjected to antimicrobial treatments using transmission (Raafat et al., 2008) and/or scanning electron microscopy (Kwon et al., 2003); cell membrane integrity, e.g. the uptake of fluorescence probe NPN into damaged cell membrane (Helander et al., 1998), study of leakages of different intracellular constituents, e.g. phosphate ions (Lambert et al., 2001), potassium ions (Lambert et al., 2001), ATP (Helander et al., 1998, Ultee et al., 1999), protein (Kwon et al., 2003, Helander et al., 1998) and "A_{260} absorbing materials", i.e. nucleic acid (Chen and Cooper, 2002, Je and Kim, 2006); and measurement of relativity of trans-membrane potential, which is suggested to be important for ATP synthesis (Ultee et al., 1999).

In the study of Kwon et al. (2003), the bactericidal effect of cinnamaldehyde and its mode of action on *Bacillus cereus* were investigated, involving the use of protein dye to determine the leakage of protein and scanning electron microscope to observe the cell morphology. Treatment of exponential phase *B. cereus* showed a strong inhibition of cell separation but no significant protein leakage was observed and did not cause cell autolysis even though the cells were already dead.

Wendakoon and Sakaguchi (1995) examined the effect of water and ethanol extract of a range of different spices, including black pepper, cumin, cinnamon, cloves, mustard and sage, etc. on the enzyme activity of cell free extract prepared from *Enterobacter aerogenes*, and found that some of them are effective in inhibiting the decarboxylase enzymes.

Lambert *et al.* (2001) found that oregano oil and its two principle components, thymol and carvacrol damage membrane integrity, which further affects pH homeostasis and

equilibrium of inorganic ions, potassium ions and phosphate ions of *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Gustafson *et al.* (1998) and Cox *et al.* (1998) found that tea tree oil stimulated autolysis, caused loss of electron dense material, coagulation of cell cytoplasm, formation of extracellular blebs, inhibit glucose-dependent respiration and stimulates leakage of intracellular potassium ions in *E. coli.*

According to two separate studies performed by Helander *et al.* (1998) and Ultee *et al.* (1999), carvacrol showed antimicrobial effects on both Gram-negative bacteria, *Escherichia coli* and Gram-positive, *Bacillus cereus* respectively but exerted different mechanisms of action. Helander *et al.* (1998) found that carvacrol changed the membrane integrity and caused leakage of cellular material including ATP out of the cell, though the amount of extracelluar ATP was low, it was significant; while Ultee *et al.* (1999) found that after the treatment of carvacrol, the ATP concentration inside the cells declined but there was no proportional leakage of ATP to the external environment. It is possible that carvacrol might have reduced the rate of ATP synthesis or increased ATP hydrolysis. Such differences in the mode of action of carvacrol may be due to the fact that different bacteria were used in the two studies.

The previously described studies generally involve the use of stationary phase bacterial cultures and antimicrobial treatments at MIC levels. Apart from these, another approach to the study of the molecular interaction between the antimicrobials and bacterial cultures is to evaluate the adaptation mechanisms of bacterial cultures growing at sub-lethal concentration of antimicrobials, for example proteomic studies (Cho et al., 2007), which is the study of protein expression and repression in bacterial cultures under the influence of antimicrobials at a sub-lethal concentration. In a proteomic analysis of *E. coli* exposed to green tea polyphenols carried out by Cho et al. (2007), nine upregulated proteins were successfully identified, including proteins connected with cellular defence mechanisms, e.g. DNA gyrase subunit A, superoxide dismutase and multidrug resistance protein K. In contrast to the control growing under normal condition, eight proteins were

decreased markedly or not expressed at all, including proteins involved in peptide bond and amino acid synthesis, phospholipid, carbon and energy metabolism.

Another approach to the study of the mechanism of bacterial survival under a sub-lethal stressor is the use of gas chromatography to study the change of membrane lipid composition, mainly the fatty acid profile, whether unsaturated fatty acid or saturated fatty acid was dominated, in bacterial cultures (Di Pasqua et al., 2006, Di Pasqua et al., 2007, Patrignani et al., 2008). Di pasqua et al. (2006) demonstrated that when culturing pathogenic organism, e.g. *E. coli* in growth media added with low concentration (below MIC) of essential oil, e.g. carvacrol and cinnamaldehyde, the membrane fatty acid profile was changed, more unsaturated fatty acids were found in the membrane, which could give a higher degree of membrane fluidity. In contrast, when treating grown bacteria culture with a high concentration (above MIC) of essential oil for a shorter period of time (2 h of incubation), the membrane fatty acid profile was altered in different way, more saturated fatty acids were observed. The researchers suggested that the mode of antimicrobial action was against the outer cell envelope (Di Pasqua et al., 2007). Not only a antimicrobial stressor could lead to an increase of synthesis of unsaturated fatty acids in order to maintain fluidity but other factor could also contribute to such a change, e.g. low growth temperature (Evans et al., 1998), which could be regarded as a universally conserved adaptation response (Keweloh and Heipieper, 1996, Di Pasqua et al., 2006). However, the report on increase of synthesis of saturated fatty acids in cells treated with naturally occurring compounds has been limited (Di Pasqua et al., 2007).

Besides proteomic analysis and the study of membrane fatty acid profile, another approach to study the mechanism of bacterial survival is the analysis of compatible solutes, also known as osmoprotector solutes. Compatible solutes are substances to help the bacterial cultures to survive under sub-lethal osmotic pressure by balancing the osmotic pressure and helping to preserve protein function inside the cells, especially when the bacterial cultures were grown in the presence of hydrocarbons or high salinity, e.g. marine environment (Beales, 2004). Production of compatible solutes is one of the

important mechanism for hydrocarbon degrading bacteria to survive and multiply in polluted environments, thus bioremediation would be allowed (Sabirova et al., 2008). Examples of compatible solutes include glycerol, trehalose, sucrose and mannitol and their production is usually analysed using high performance lipid chromatography (Esteves et al., 2009, Canamas et al., 2007).

Apart from studying the antimicrobial mode of action using live bacterial cultures, researchers have also used model systems to study the molecular interaction between the antimicrobials (both naturally occurring compounds and synthetic antibiotics) and phospholipids of the bacterial membrane, as it has been widely accepted that most antimicrobials exert their antimicrobial effects on the bacterial membrane and membrane as it is usually the first biological structure encountered by drugs (Dennison et al., 2009, Giordani et al., 2006, Gidalevitz et al., 2003). As the biological cell membrane is very complex, while the model membrane is a one component system, which allows study of interaction between specific membrane lipid and antimicrobial compounds. The model membrane systems commonly used include liposomes (unilamellar or multilamellar), lipid bilayers (spherical or planar) and lipid monolayers (Ambroggio et al., 2005, Trombetta et al., 2005, Barzyk et al., 2009, Tamba et al., 2007).

Monolayer model membrane systems could be used to study the change of thermodynamical properties of phospholipids commonly found in bacterial culture (Maget-Dana, 1999, Simons and Vaz, 2004, Brockman, 1999). The penetration of these antimicrobials into phospholipid monolayer model membranes, as well as their interactions with lipids in mixed films could be evaluated. These studies allow the measurement of quantitative effects of antimicrobials on model membranes (Dynarowicz-Łątka et al., 2001). Different parameters could be measured and calculated from the monolayer system, e.g. kinetic study of the change of surface pressure versus time after addition of antimicrobials to the subphase under lipid monolayer (Dennison et al., 2009), the purpose of these studies was to evaluate the affinity of the antimicrobials to the lipid monolayer in a time dependent manner, as an increased surface pressure indicates that the antimicrobials are incorporating into the lipid monolayer, thus

affecting the molecular organisation packing behaviour and density of phospholipid molecules in two dimensional structure.

Apart from the kinetic study, the two parameters used in the monolayer technique, surface pressure-area isotherms and surface potential-area isotherms are also ideal for studying the interaction between antimicrobial compounds and model membrane. Surface pressure-area isotherms can be used for the calculation of compressibility moduli, which is an indicator of membrane fluidity (Sabatini et al., 2008, Wydro and Witkowska, 2009), related to the survival adaptation response (Di Pasqua et al., 2006); while surface potential of the monolayer system may reflect the actual trans-membrane potential in live bacterial cells, such trans-membrane potential has been suggested to be important for ATP synthesis (Ultee et al., 1999).

Despite many studies which have been carried out in attempts to evaluate the mode of antimicrobial action of naturally occurring compounds, the exact mechanisms of these antimicrobials remain vaguely defined (Burt, 2004). The mechanisms of antimicrobial action of the naturally occurring compounds are important because those potentially effective compounds might be used in combinations to exert synergistic effects (Gill and Holley, 2004).

1.3.13 Possible synergistic effects

Synergistic effect refers to the interaction of two or more compounds, e.g. drugs, when combined, would produce a total effect that is greater than the sum of the individual compounds. Additive effect refers to the combined effect of two or more chemicals, which is equal to the sum of their individual effects. Antagonistic effect refers to the interaction of two or more chemical substances whose overall effect is less than the sum of the effects of the substances.

Synergistic study is important and is also a common tool in many life science areas especially in medical and health research for pharmaceutical drug evaluation and development. Compounds having different modes of antimicrobial action might be

applied in combinations to decrease the amount of each compound to be used. Examples of synergistic study include combinations of different antibiotics (Miranda-Novales et al., 2006), combinations of antibiotics and natural antimicrobials (Shahverdi et al., 2007, Hemaiswarya and Doble, 2009, Kim et al., 2009), combinations of different natural antimicrobials (Nazer et al., 2005), and combinations of different natural antimicrobials and physical treatments, e.g. heat and refrigeration (Valero and Francés, 2006) and different water activity and pHs (Santiesteban-Lopez et al., 2007). Synergistic study within the food industry is important because the existence of synergy may lead to reduction of use of antimicrobials that can help in addressing the consumer concerns regarding chemical preservatives (Santiesteban-Lopez et al., 2007) and sensory acceptance.

As important as MICs, synergistic study can determine the minimum amount of compounds required to maintain an effective microbial activity, thus the amount and cost of required for a treatment will be lowered. Moreover, synergism may also be helpful for tackling the problem of conventional drug resistance. Research studies have demonstrated that impermeability of bacterial plasma membrane could be responsible for the resistance of aminoglycoside. A synergistic study of conventional antibiotics plus natural plant product performed by Kim et al. (2009) suggested that altering the membrane stability by application of naturally occurring compounds might allow the antibiotics to restore the effectiveness to the methicillin-resistant *Staphylococcus aureus*. An additional advantage to this is that when administering the drug at lower concentration, the undesirable side effects may also be lowered (Rosato et al., 2007).

Besides the potential to tackle the drug resistant bacteria, synergistic study could also have an impact on the control of Gram-negative bacteria, which are generally more resistant to antimicrobial treatments, due to the presence of lipopolysaccharides in the outer membrane. Zhou et al. (2007) demonstrated the synergistic effect of combination of chelating agent EDTA and naturally occurring compounds against *Salmonella typhimurium* and the possible reason was the chelating agents disrupted the outer membrane thus enhanced the sensitivity of *S. typhimurium* to the naturally occurring

compounds. In addition to chelating agent, they also demonstrated the synergistic effect of combination of acetic acid and naturally occurring compounds and suggested the possible mechanism was the presence of acetic acid might allow naturally occurring compounds to exist in a form that is more easily available to the cells.

Another example was the synergistic effect of the combination of non-bactericidal emulsifying agent (linolenic acid) and the naturally occurring compounds, possibly through the increase in the adsorption of linolenic acid, an antimicrobial fatty acid derived from plants, to cell surface and penetration into the bacterial cells (Lee et al., 2002). Synergistic studies of combinations of different naturally occurring compounds have been reported by Nazer et al. (2005), though better amplification of inhibition was observed, the researcher concluded that was merely cumulative effect. However, this finding was still considered interesting as the amount of antimicrobial compounds used was lowered.

1.4 Aims and objectives

The aims of this study were to evaluate the antimicrobial activity of a range of naturally occurring compounds against a number of selected food and animal pathogens and to investigate the possible mode of antimicrobial action of the active natural compounds which could be used in agricultural animal and food productions.

The objectives of this research study could be classified into three main categories.

Firstly the determination of possible antibacterial activities of naturally occurring compounds against microorganisms relevant to food safety, i.e. *Escherichia. coli* (*E. coli*), *Clostridium sporogenes* and *Mycobacterium avium* subspecies *paratuberculosis* (*Map*).

Secondly the determination of the possible synergistic effects of combinations of naturally occurring antimicrobial compounds and other antimicrobial substances e.g. amphibian peptides and extracts of medicinal plants and herbs on the inhibition of

growth of *Map* NCTC 8578 using both macrobroth dilution susceptibility test and microbroth dilution susceptibility test

Thirdly the determination of possible antimicrobial modes of action of the active compounds. Two approaches were used. The first was undertaken through a series of experiments including study of autolysis, determination of phosphate concentration, amount of soluble protein and nucleic acid in extracellular environment, determination of intra- and extracellular ATP concentrations and absorbance scan of culture supernatant after antimicrobial treatment, which involve the use of live bacterial cultures. The second approach was to study the interactions of naturally occurring antimicrobial compounds on monolayers (model membranes) composed of phospholipids commonly found in bacterial membrane, thus to determine the possible mode of action of these naturally occurring compounds.

2 Antibacterial Activities of Naturally-Occurring Compounds against *Escherichia coli* and *Clostridium sporogenes*

2.1 Introduction

Escherichia coli (*E. coli*) are Gram-negative straight rod-shaped bacteria. They are commonly found in the gut, mainly the lower part of the intestine of humans and other warm-blooded animals. Most strains of *E. coli* are harmless. However, some strains such as enterohaemorrhagic *E. coli* (EHEC) produce enterotoxins, known as enterotoxigenic strains, can cause severe foodborne disease (WHO, 2005). EHEC infections are important foodborne diseases, which have emerged over the last decades.

Clostridium perfringens (*Cl. perfringens*) is an obligatory anaerobe and is capable of forming spores under adverse growth conditions, e.g. when nutrients are depleted. Similar to *E.* coli, its optimum growth temperature is between 30-37°C (Cato et al., 1986), which is very close to the basal body temperature of mammals. It is both a human and animal disease pathogen, causing foodborne illness in humans and necrotic enteritis in chickens (Mitsch et al., 2004). *Cl. perfringens* is ubiquitous, commonly found in soil, sewage, decaying vegetation, animal and plant products, the intestines of humans, other vertebrates and in wounds or soft tissue infections of humans and animals (Cato et al., 1986). Necrotic enteritis is the inflammation of the intestinal tract. Transmission of *Cl. perfringens* is through faecal-oral route between animals. The most often presented clinical sign is a sudden increase in mortality, diarrhoea might also be observed, thus affecting the performance of the broilers and subsequently causing economic losses of producers (Meriden Animal Health Limited, 2010). Consumers could be infected through eating undercooked or contaminated chicken. The most effective way to prevent necrotic enteritis is to add antibiotics in broiler feeds.

Antibiotics have been routinely incorporated into broiler feed as growth promoters to increase feed conversion efficiency and decrease mortality (Ross Tech, 1999). However,

such use maybe attributed to the development of antibiotic resistant pathogens in animals, which could be transferred from animals to humans via the food chain (Pfaller, 2006, Turnidge, 2004). Concerns of the extensive use of chemical drugs in farm animals have been raised by the public (White and McDermott, 2001), as residues of drugs or their metabolites might be ingested by humans via the food chain (Guo et al., 2004a). Consumers in Europe are demanding antibiotic-free livestock production (BBC, 1998). The increasing concerns of developing antibiotic resistance in animals which may ultimately transfer to humans via the food chain eventually led to an EU-wide ban on the use of all growth promotion antibiotics in food animal production the new regulation 1831/2003/EC came into force in January 2006 in the Europe (OJEU, 2003). As a result, the agricultural industries have been actively searching for natural, safe and effective alternatives, which can be used in food animal production (Schöne et al., 2006).

Medicinal plants and natural remedies have been traditionally used in folk medicine in many parts of the world for thousands of years. Many plant-derived bioactive compounds have continually been discovered and characterised (Fitzgerald et al., 2004, Gill and Holley, 2004, Helander et al., 1998, Ultee et al., 1999). They are considered to be ideal for study as most of them are 'generally recognised as safe' (GRAS) (Kabara, 1991). There is enormous scope for exploring these naturally-occurring compounds as potential 'neutraceuticals' as many of these have already shown very promising antimicrobial activities. For instance, some tea extracts have been found to be more effective against several bacteria than currently used antibiotics at similar concentrations (Friedman et al., 2006b). Other compounds such as phenols, essential oils and some of their purified and isolated components are also effective against some food borne pathogens (Friedman et al., 2002, Friedman et al., 2003, Friedman et al., 2004b) and some of them may have an effect on antibiotic-resistant pathogens (Friedman et al., 2004a).

Studies have shown that when capsaicin, cinnamaldehyde and carvacrol were incorporated into chicken diets based on maize or wheat and barley, body weight was not enhanced but feed conversion index was significantly improved. This possibly be

due to the better utilisation of nutrients within the feed (Jamroz et al., 2005). Naturally occurring compounds derived from animal origins for example frog skin peptides which aid in the host defence mechanism have also received increasing attention. Recent studies have demonstrated that amphibian peptides exert significant antimicrobial activities against bacteria and fungi, including Methicillin-resistant *Staphylococcus aureus* (MRSA) (Pál et al., 2006).

In the present study, *Clostridium sporogenes* (*Cl. sporogenes*) was selected for use as a surrogate of *Cl. perfringens* as this pathogenic organism is listed in the Schedule 5, Anti-terrorism, Crime and Security Act 2001 (OPSI, 2007). Another surrogate organism, *Escherichia coli* K12 (Category 1) was used as a surrogate for the pathogenic *E. coli* O157:H7 (which requires Category 3 facilities). These two surrogates of animal and human pathogens were selected because they are very different in terms of biological structure and are also the major food pathogens that have severe economic impacts on agricultural food industries as well as the public health sector.

2.2 Materials and methods

2.2.1 Test compounds

The following compounds were originally obtained from Sigma (St. Louis, MO, USA): 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, caffeic acid, capsaicin (natural), carvacrol, chitosan from crab shells (min. 85% deacetylated), chlorogenic acid hemihydrates, *trans*-cinnamaldehyde, *trans*-cinnamic acid, citral (*cis* + *trans*), gallic acid, garlic oil, geraniol, methyl cinnamate, onion oil and vanillic acid. The purity levels of these compounds ranged from 95 to 99.9% according to the manufacturers. Cinnamon oil (Cassia) was originally obtained from Yerba Buena Co. (Berkeley, Calif. USA). Oregano oil (Origanum) was originally obtained from Lhasa Karnak Herb Co. (Berkeley, Calif. USA). Their purity

levels were not specified. Apple E (concentrated apple polyphenols) was originally obtained from Apple Poly LLC (Morrill, NE, USA); the purity level was approximately 82%. Green tea polyphenols were originally obtained from LKT Laboratories, Inc. (St. Paul, MN, USA); the purity level was not specified. Garlic powder and onion powder (cooking ingredients) were originally obtained from McCormick & Company Inc. (Sparks, MD, USA). Their purity levels were not specified. The above compounds were gifts from Dr Mendel Friedma, Western Regional Research Center, U.S. Department of Agriculture, Albany, California, USA. Gentamycin and ampicillin (Sigma) were used as positive control antibiotics as they were known to be active against *E. coli* and *Cl. sporogenes* respectively. Amphibian peptide QUB 2841.38 was obtained from the School of pharmacy, Queen's University Belfast, UK. Growth media: nutrient broth, nutrient agar, brain heart infusion broth and brain heart infusion agar were obtained from Oxoid (Unipath Ltd, Basingstoke, UK).

2.2.2 Preparation of test compounds for bactericidal assay.

In order to evaluate the antimicrobial activity of the test compounds, the bactericidal assay developed by Friedman et al. (2002) was adopted in the study. Many of the plant essential oils and compounds purified from essential oils are immiscible in the aqueous buffers used in bactericidal assays. Ethanol was therefore used to prepare the stock suspension of the oil compounds.

A stock solution of, 50 mg/ml for solid compounds or 50 µl/ml for oil compounds was prepared by suspending each test compound in absolute ethanol, except chitosan, which was prepared as a 10 mg/ml stock suspension in 1 M acetic acid as suggested by the manufacturer. The stock solutions were stored in aluminium foil-wrapped bottles at 4°C. Gentamycin and ampicillin were prepared as 1 mg/ml stock suspension in 50 mM sterile PBS, pH 7.0 and aliquots were stored at -70°C. As a high percentage of ethanol could be

bactericidal, the amount of ethanol being added to the growth medium was kept low and constant in all the tests, in order to minimize adverse effect on recovery of the strains.

Before diluting the test compounds in buffer, each stock solution was vortexed vigorously for 10 s. Twelve microliters of each stock solution was pipetted immediately to 588 µl of 50 mM PBS (pH 7.0). Thus, the concentration of the test compound in the first dilution was 1 mg/ml and ethanol was 2% (vol/vol). An aliquot (200 µl) of this compound suspension was drawn immediately after vortexing and added to 400 µl of PBS containing 2% (vol/vol) ethanol for standardizing the concentration of ethanol in all dilutions for a 1:3 dilution. The mixture was vortexed vigorously for 10 s before an aliquot was taken and added to the next eppendorf tube. Samples were serially diluted five times, for six dilutions, 1000, 333.3, 111.1, 37.0, 12.3 and 4.1 µg/ml.

For chitosan, 60 µl of stock suspension was used instead of 12 µl as the stock was prepared in acetic acid. The positive control antibiotics were diluted to 333.3, 111.1, 37.0, 12.3, 4.1 and 1.4 µg/ml. Ethanol 2% (vol/vol) and 100 mM acetic acid were used as negative controls. Preliminary experiments confirmed that 2% (vol/vol) ethanol did not cause adverse effect on the recovery of both bacterial strains.

2.2.3 Bacterial strains and growth condition.

Escherichia coli (*E. coli*) K12 NCTC 10538, *Clostridium sporogenes* (*Cl. sporogenes*) NCTC 8594 were obtained from the National Collection of Type Cultures, Colindale, London. Both strains were maintained in Cryobank vials (Mast Group Ltd, Merseyside, UK) at -70°C prior to experiment.

For culturing *E. coli*, a cryobead was inoculated into 5 ml of nutrient broth (Oxoid, Unipath Ltd, Basingstoke, UK) and incubated for 24 h at 37°C with gentle shaking (100 rpm). After reviving the culture, the broth was streaked onto nutrient agar (Oxoid, Unipath Ltd, Basingstoke, UK) plate and incubated overnight at 37°C to produce isolated colonies. A single colony was used to inoculate 5 ml of nutrient broth (Oxoid,

Unipath Ltd, Basingstoke, UK) and incubated for 19 h at 37°C with gentle shaking (100 rpm) for bactericidal assay.

For culturing *Cl. sporogenes*, a cryobead was inoculated into 5 ml of brain heart infusion (BHI) broth (Oxoid, Unipath Ltd, Basingstoke, UK) and incubated statically at 37°C for 24 h in the anaerobic cabinet. After reviving the culture, the broth was streaked onto brain heart infusion agar plate (Oxoid, Unipath Ltd, Basingstoke, UK) and incubated at 37°C overnight to produce isolated colonies. A single colony was used to inoculate 5 ml of Brain heart infusion (Oxoid, Unipath Ltd, Basingstoke, UK) and incubated statically at 37°C for 20 h for bactericidal assay. Both of the culture media, broth and agar for *Cl. sporogenes* were pre-conditioned in the anaerobic cabinet overnight before use. The atmosphere in the anaerobic cabinet was maintained with a cylinder of anaerobic gas mixture 10%CO_2:10% H_2:80% N_2 (BOC Special Products Limited, Guildford, UK).

2.2.4 Preparation of bacteria for bactericidal assay.

One milliliter of broth culture of *E. coli* or *Cl. sporogenes* was added to a 1.5-ml eppendorf tube, the bacteria were pelleted by centrifugation at 550 g for 4 min. After the removal of the supernatant, 1 ml of sterile 50 mM PBS was added to the pellet and the pellet was resuspended by gentle aspiration in and out of a transfer pipette. The PBS used for preparation of bacterial suspension of *Cl. sporogenes* was pre-conditioned in the anaerobic cabinet overnight before use. The optical density (OD) of the resuspended pellet was determined using a cell density meter (WPA Biowave CO8000, Biochrom Ltd, Cambridge, UK) set at 600 nm. The OD_{600} of the bacterial suspension was then adjusted to approximately 0.8 with the addition of sterile PBS. Ten microliters of the resultant bacterial suspension was added to 990 µl of PBS (1: 100), then 40 µl of the 1:100 dilution was further diluted in 10 ml of PBS for *E. coli* (final dilution = 1:25,000 from the adjusted suspension) and to 1:2,000 for *Cl. sporogenes.*

2.2.5 Bactericidal assay

The assay to assess the antimicrobial activities of naturally occurring compounds was carried out as described by Friedman et al. (2002) with slight modifications.

One-hundred microliters of the 6 dilutions of test compound prepared as described previously were pipetted to a U-bottom 96-well plate (Sarstedt Ltd, Leicester, UK,) in duplicate, negative control 2% (vol/vol) of ethanol was included in each plate and 100 mM acetic acid was included in the reaction plate containing chitosan. To each well, 50 µl of the diluted bacterial suspension was added. For the assay of *Cl. sporogenes*, after transferring the dilutions of test compounds to the 96-well plates, the plates were placed in the anaerobic cabinet for 1 h to eliminate the oxygen dissolved in the PBS during the dilution step, before the addition of the bacterial suspension. After the addition of the bacterial suspension, the 96-well plate was vortexed thoroughly but carefully to avoid spillage. The final concentrations of each test compound in the 96-well plate were 666.7, 222.2, 74.1, 24.7, 8.2 and 2.7 µg/ml, ethanol present in each well was 1.3% (vol/vol) and the concentration of acetic acid was 66.7 mM after the addition of bacterial suspension. The 96-well plates were then incubated at 37°C with gentle shaking (100 rpm) for 1 h (and in the anaerobic cabinet for *Cl. sporogenes*).

Following incubation, a 10 µl aliquot from each well was spotted at the top of a square Petri plate containing nutrient agar (for *E. coli*) or brain heart infusion agar (for *Cl. sporogenes*). The plate was tilted and tapped gently to facilitate the movement of the sample to the bottom of the plate. Each square Petri plate could accommodate six 10 µl samples, which were spaced evenly across it. There were approximately 50 to 100 colony forming unit (cfu) in the spotted 10 µl sample from the control well. The square Petri plates were left on the bench for approximately half an hour until the sample liquid dried, then the plates were incubated upside down at 37°C overnight (and in the anaerobic cabinet for *Cl. sporogenes*) for cfu enumeration. Usually, 50 to 100 cfu were present in the negative control. The experiments were repeated twice with two separately prepared bacterial suspensions for each strain.

2.2.6 Minimum Inhibitory Concentration (MIC) estimation

The numbers of cfu counted for the six concentrations of the test compounds, the negative controls and the positive controls were recorded using a Microsoft Excel

spreadsheet. The number of cfu at each dilution was matched with the average negative control value to determine the percentage of bacteria killed per well. The percentage of bacteria killed was plotted against the concentration of the test compound; and the concentration of the test compound in the reaction well resulting in a 50% decrease in the number of cfu (BA_{50}) was determined. BA_{50} was used to determine the relative potencies and the ranking of bactericidal activities. The lower the BA_{50} value, the higher the bactericidal activity.

2.3 Results

Bactericidal activities of the compounds tested are presented in the following figures (figures 2.1-2.12) using the percentage kill plotted against the concentration of test compounds. As suggested by Friedman et al., the minimal inhibitory concentration (MIC) was defined as bactericidal activity 50 (BA_{50}), i.e. the concentration required to decrease 50% of the bacterial colony forming units (cfu). All MIC values were determined from the above plots and are presented in table 1.

2.3.1 Evaluation of antibacterial activity against *E. coli*

Among those 22 essential oils and their isolated constituents, 18 of them were found to be active against *E. coli*. Of those 18 antibacterial compounds, 7 exerted their activities at a relatively low concentration (<100 μg/ml). They were, in order of potency, 2,5-dihydroxybenzaldehyde, apple E polyphenols, gallic acid, green tea polyphenols, garlic powder, onion powder and oregano oil. Neither of the two naturally occurring compounds from animal origins showed antibacterial activity: chitosan was not active against *E. coli* even at the highest concentration (666.7 μg/ml) tested; amphibian peptide (QUB 2841.38) did not show activity against *E. coli* after one hour of incubation at any concentrations tested. A low level of activity was observed after 3 to 4 hours of incubation at the highest concentrations tested (32 μg/ml).

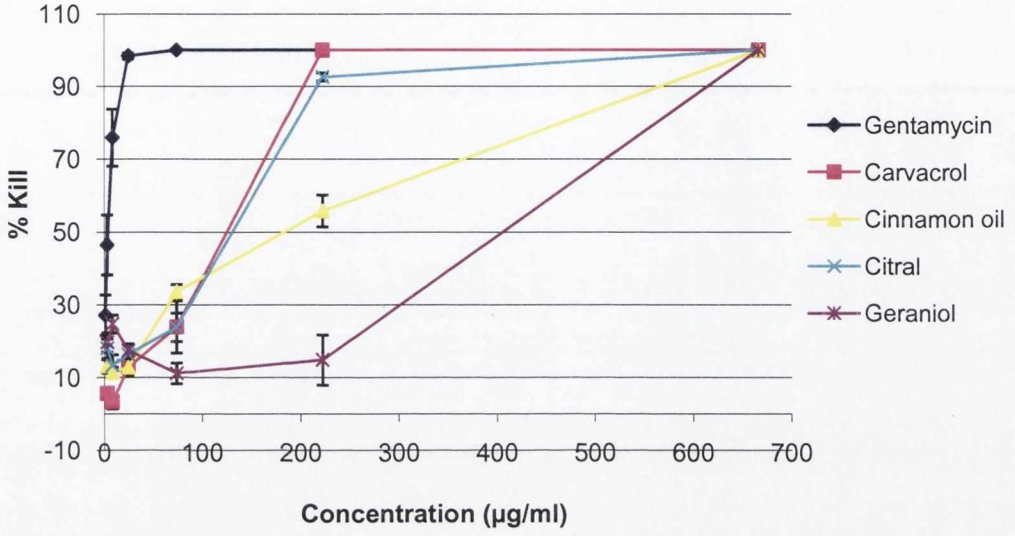


Figure 2.1 Dose response plots of concentration (µg/ml) versus % kill of bacteria for positive control antibiotic gentamycin, compounds carvacrol, cinnamon oil, citral and geraniol against *E. coli* K12. Vertical bars indicate standard error of the mean (SEM).

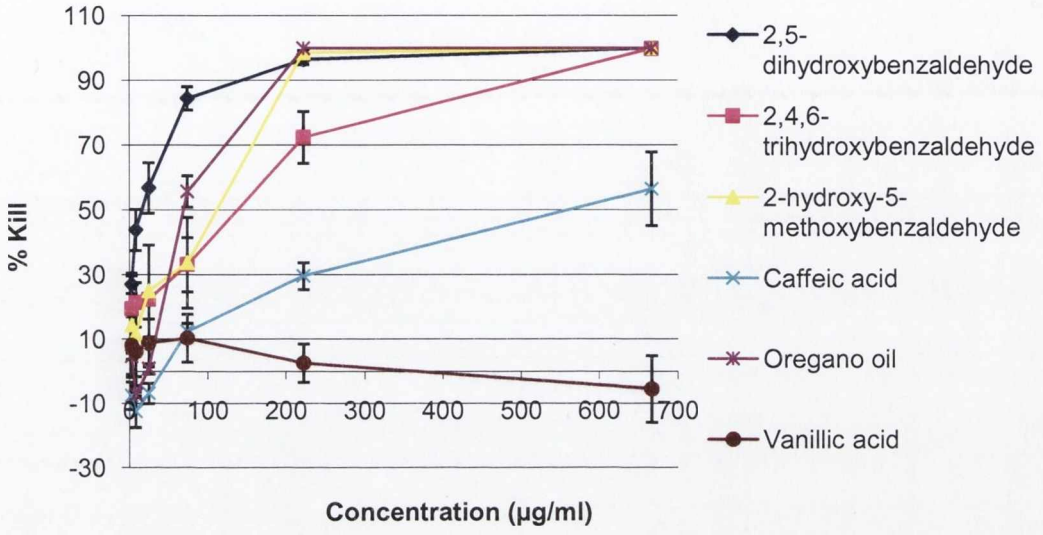


Figure 2.2 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, caffeic acid, oregano oil and vanillic acid against *E. coli* K12. Vertical bars indicate standard error of the mean (SEM).

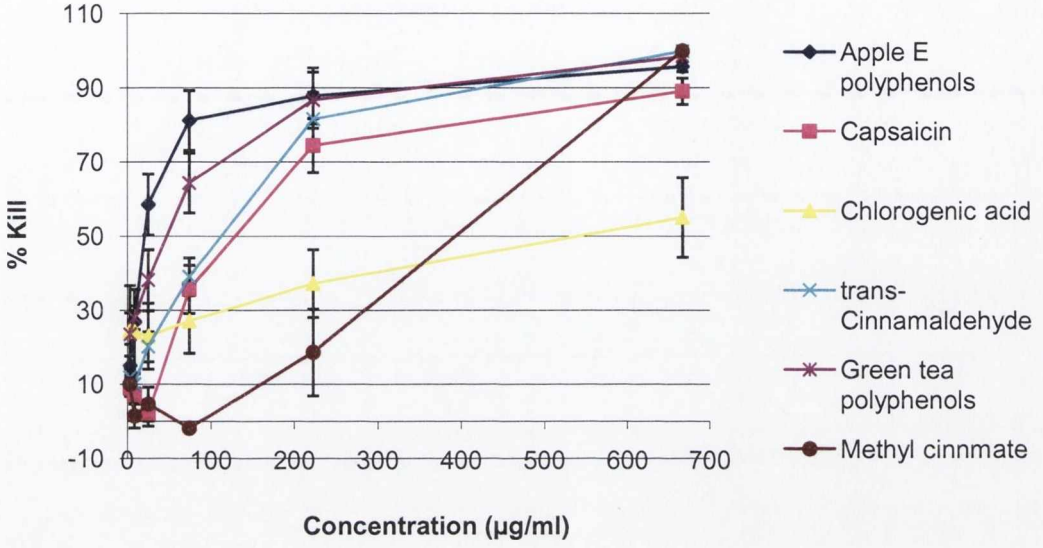


Figure 2.3 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds Apple E, capsaicin, chlorogenic acid, cinnam-aldehyde, green tea polyphenols and methyl cinnamate against *E. coli* K12. Vertical bars indicate standard error of the mean (SEM).

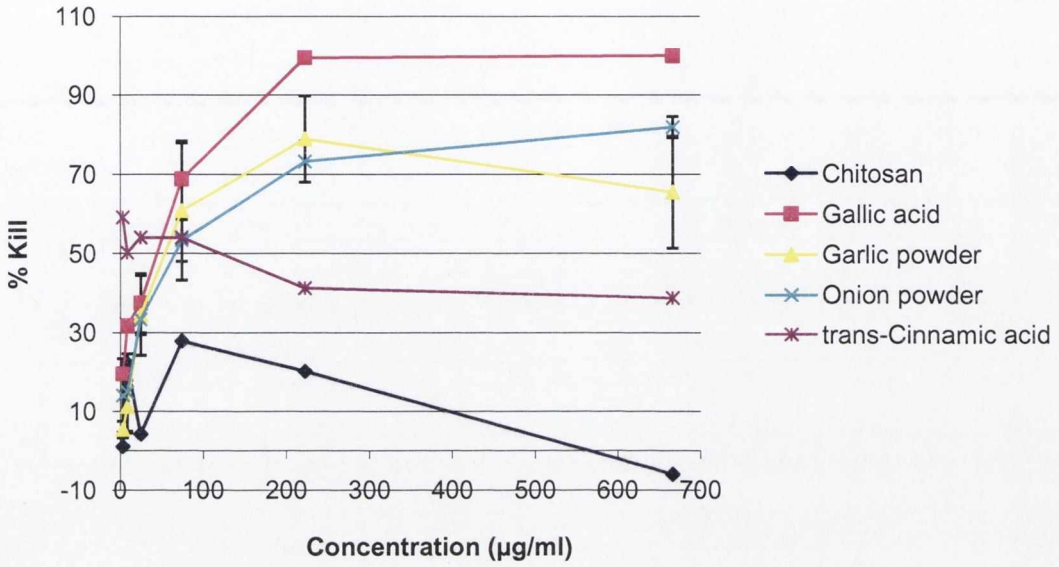


Figure 2.4 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds chitosan, gallic acid, garlic powder, onion powder and *trans*-cinnamic acid against *E. coli* K12. Vertical bars indicate standard error of the mean (SEM).

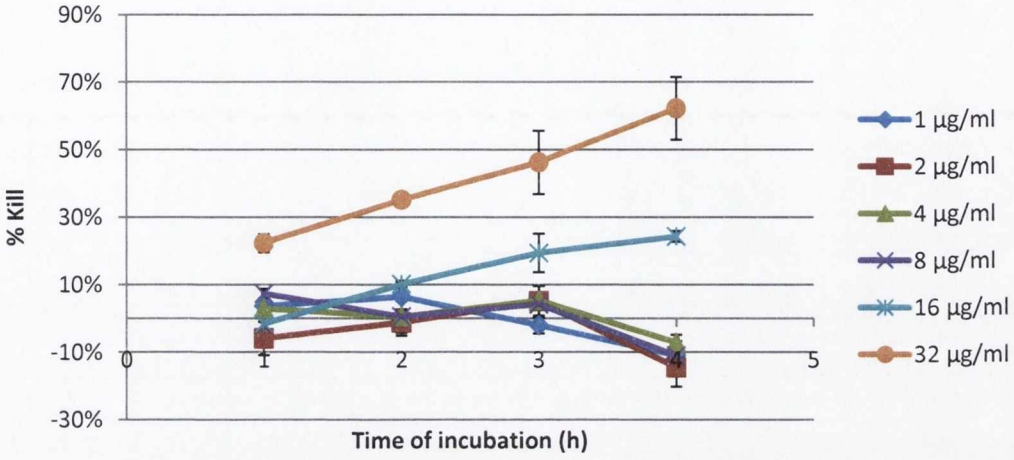


Figure 2.5 Effect of different concentrations (µg/ml) of amphibian peptide QUB 2841.38 on the recovery of *E. coli* K12 on nutrient agar plate. Vertical bars indicate standard error of the mean (SEM).

2.3.2 Evaluation of antibacterial activity against *Cl. sporogenes*

Among the 22 essential oils and their isolated constituents tested, 15 were found to be antibacterial and 5 of these exerted their bactericidal activities at a relatively low concentration (<100 μg/ml). These were green tea polyphenols, apple E polyphenols, oregano oil, onion oil and carvarcol. The MIC of positive control ampicillin was 19 μg/ml.

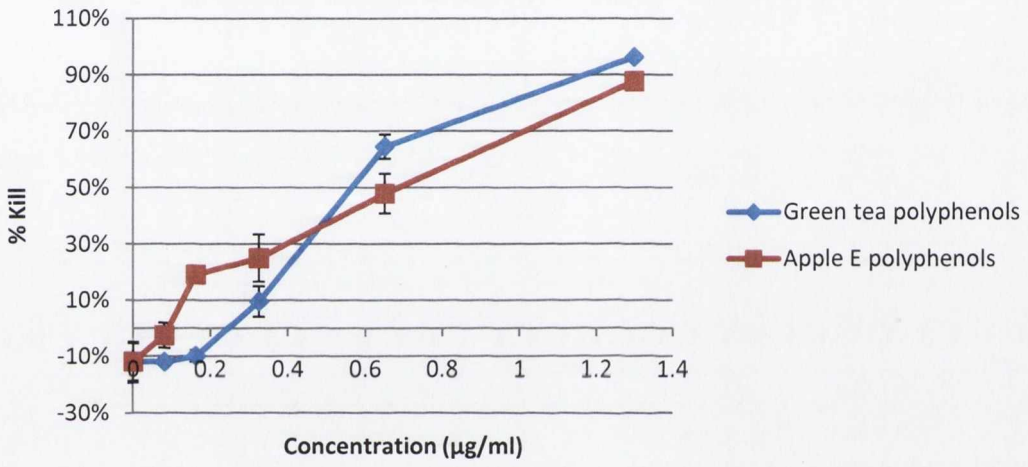


Figure 2.6 Dose response plots of concentration (μg/ml) versus % kill of bacteria for compounds green tea polyphenols and apple E polyphenols against *Cl. sporogenes*. Vertical bars indicate standard error of the mean (SEM).

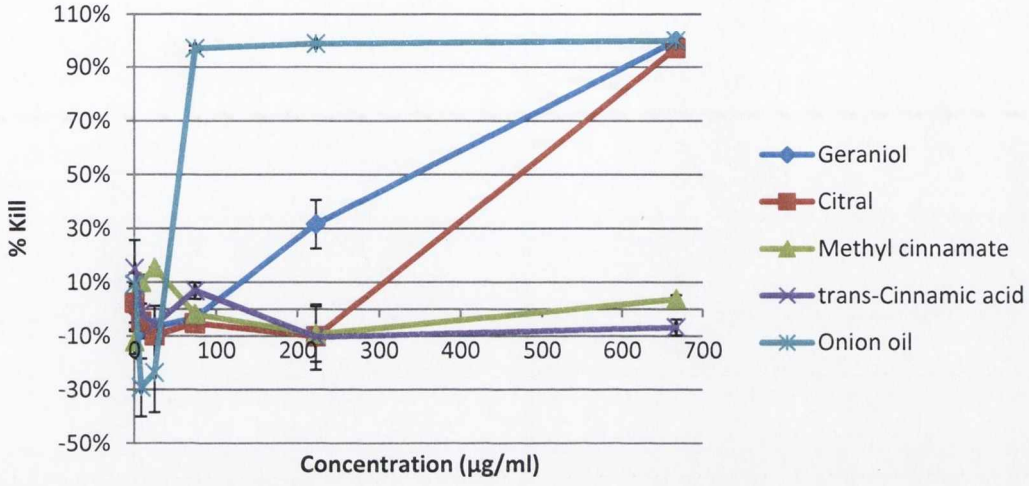


Figure 2.7 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds geraniol, citral, methyl cinnamate, *trans*-cinnamic acid and onion oil against *Cl. sporogenes*. Vertical bars indicate standard error of the mean (SEM).

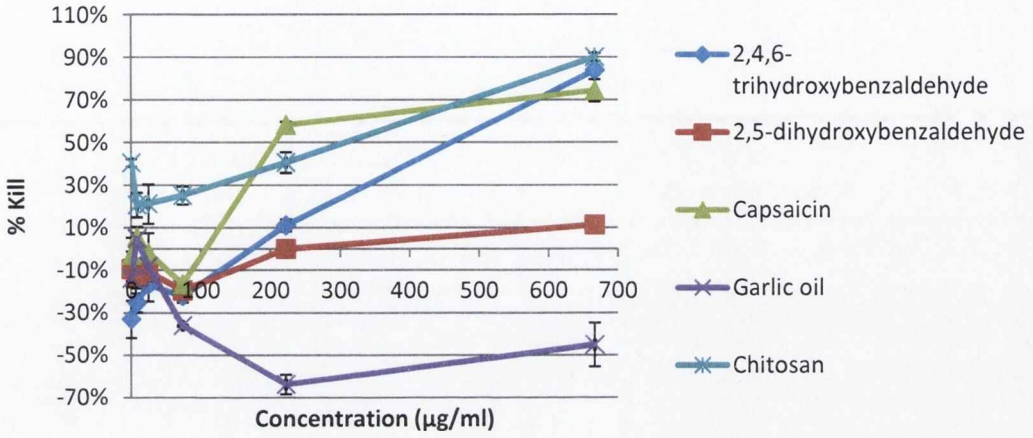


Figure 2.8 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, capsaicin, garlic oil and chitosan against *Cl. sporogenes*. Vertical bars indicate standard error of the mean (SEM).

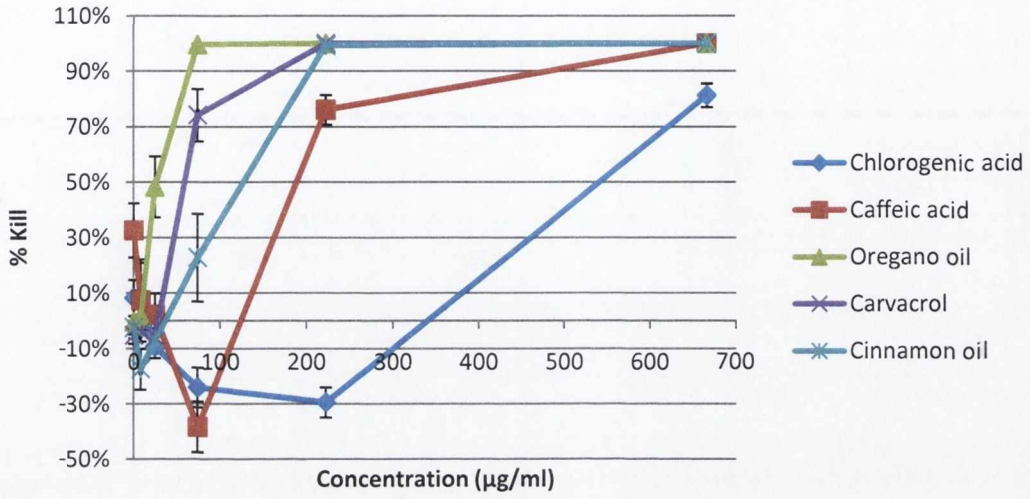


Figure 2.9 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds chlorogenic acid, caffeic acid, oregano oil, carvacrol and cinnamon against *Cl. sporogenes*. Vertical bars indicate standard error of the mean (SEM).

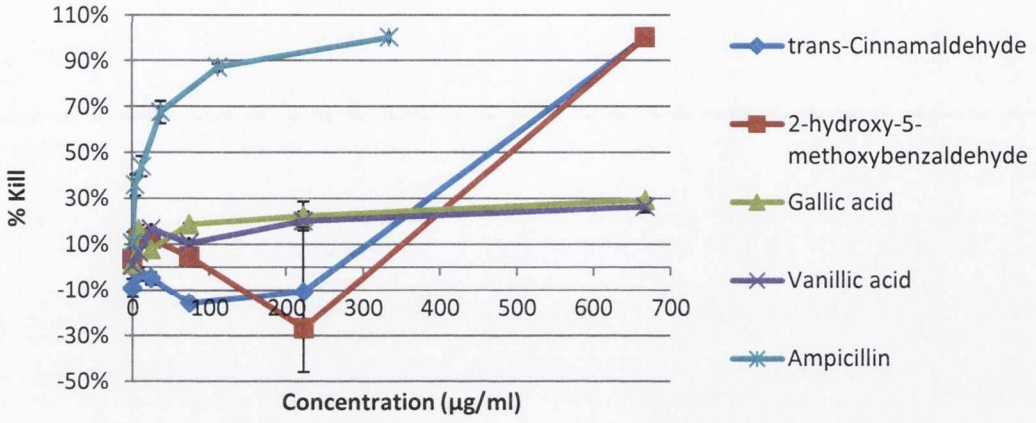


Figure 2.10 Dose response plots of concentration (µg/ml) versus % kill of bacteria for compounds cinnam-aldehyde, 2-hydroxy-5-methoxybenzaldehyde, gallic acid, vanillic acid and positive control antibiotic ampicillin against *Cl. sporogenes*. Vertical bars indicate standard error of the mean (SEM).

Table 2.1 Minimum Inhibitory Concentration (MIC) (μg/ml) of naturally-occurring compounds tested for activity against *E. coli* K12 and *Cl. Sporogenes.*

Test compounds	MIC (μg/ml) for:	
	E. coli	*Cl. sporogenes*
Gentamycin (positive control)	3.0	nt[a]
Ampicillin (positive control)	nt	19
2,5-dihydroxybenzaldehyde	16	–[b]
apple E polyphenols	20	0.68
gallic acid	45	–
green tea polyphenols	46	0.57
garlic powder	54	–
onion powder	66	–
oregano oil	70	26
2-hydroxy-5-methoxybenzaldehyde	112	491
cinnam-aldehyde	112	466
carvarcol	124	59
capsaicin	128	206
citral	130	472
2,4,6-trihydroxybenzaldehyde	139	459
cinnamon oil	182	127
methyl cinnamate	392	–
geraniol	450	341
chlorogenic acid	538	541
caffeic acid	559	188
vanillic acid	–	–
trans-cinnamic acid	–	–
chitosan	–	306
onion oil	–	55
garlic oil	–	–
amphibian peptide QUB 2841.38	32[c]	nt

[a] The antibacterial activity of this compound was not tested against that strain.

[b] Inhibition of strain was not shown in the presence of the highest concentration of compound tested.

[c] The MIC value of this peptide was obtained after 4 h of incubation, while MIC values of other compounds were obtained after 1 h of incubation.

2.4 Discussion

2.4.1 Evaluation of antibacterial activity against *E. coli*

The naturally occurring compounds tested in the present study could be classified into three groups: naturally occurring compounds from plant origins, e.g. essential oils and their isolated constituents; naturally occurring compounds from animal origins, e.g. chitosan, a component found in the shell of aquatic species of crab; and an amphibian peptide.

2,5-dihydroxybenzaldehyde had the lowest MIC (16 μg/ml) against *E. coli* among all the compounds tested. The MIC of positive control gentamycin was 3.0 μg/ml. Benzaldehydes 2-hydroxy-5-methoxybenzaldehyde and 2,4,6-trihydroxybenzaldehyde were also active against *E. coli* but at a relatively higher concentration, which was 112 and 139 μg/ml respectively. Polyphenols, apple E and green tea were active against *E. coli* at low concentrations, which were 20 and 46 μg/ml respectively. One of the organic acids, gallic acid, was also active at a low concentration of 45 μg/ml, while the others chlorogenic acid, caffeic acid, vanillic acid and *trans*-cinnamic acid were active at relative high concentrations >500 μg/ml or did not show activity even at the highest concentration tested (666.7 μg/ml). Cooking ingredients garlic and onion powder also showed activities at low concentrations, which were 54 and 66 μg/ml respectively. The chemical compositions of both products were not specified by the manufacturer, the main active constituent responsible for the antibacterial activity needs to be identified. Essential oils and their main components showed activities at relatively low to moderate concentrations, except geraniol, which required a relatively high concentration (450 μg/ml) against *E. coli*. The MIC of oregano oil was the lowest, 70 μg/ml, its main constituent carvacrol was 124 μg/ml. The difference in MIC between these two compounds might be due to other constituents in oregano oil which were also antibacterial and these components may have exerted their activities together against *E. coli*. The MIC of cinnamon oil and its main constituent *trans*-cinnamaldehyde was 182 and 112 μg/ml respectively. Different from oregano oil and carvacrol, *trans*-cinnamaldehyde was more active than cinnamon oil, which suggested that *trans*-

cinnamaldehyde may be the main active component responsible for the antibacterial activity against *E. coli*, while other components in cinnamon oil were not as active as *trans*-cinnamaldeyde.

The bactericidal assay used in this study was adopted from Friedman et al. (2002), which was a new assay developed by their research team, several compounds, e.g. cinnamaldehyde, cinnamon oil, carvacrol, oregano oil, geraniol, 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde, tested in our study had also been tested by Friedman et al. (2003) already. Comparing the results of Friedman et al. with the results of the current study, the compounds tested in this study have lower MICs. For example, it was found that geraniol had a MIC of 450 μg/ml, which was one-third of the concentration that Friedman et al. (2002) used in their study and 2,5-dihydroxybenzaldehyde appeared to be more active for the non-pathogenic *E. coli* in our study, approximately 26 times more so (Friedman et al., 2003). One possible reason might be that the non-pathogenic *E. coli* K12 used in our study was more susceptible to antimicrobial compounds than the pathogenic strain *E. coli* O157:H7 used in Friedman et al.'s studies.

Comparison of other antimicrobial studies on naturally occurring compounds performed using different assays revealed that the MIC values obtained from the current study were still lower. For instance, Hammer et al. (1999) reported a MIC value of 0.12% (vol/vol) for oregano oil to inhibit *E. coli*, while MIC value of oregano oil in this study was 0.007% (vol/vol) (70 μg/ml). The probable reason for such a difference was that Hammer et al. incorporated oregano oil into the agar medium that contained Tween 20 to facilitate solubility of oil, and the complex ingredients contained in the growth medium might affect the antibacterial activity of the oil. Another study carried out by Chang et al. (2002) demonstrated complete inhibition of *E. coli* within 24 h in the presence of 10 mg/ml of crude Korean green tea extract. The MIC obtained by Chang et al. (2002) was high compared to the MIC of green tea polyphenols obtained in the present study, which was 46 μg/ml; such a difference might due to the crude extract of green tea also containing other non-active components and also the amount of

polyphenols may vary geographically. Furthermore, both Hammer et al. and Chang et al. determined the MIC in their study as complete inhibition, while MIC in our study was determined as 50% reduction of cfu.

Recent research has shown the prominent effect of amphibian peptides against *E. coli* (Dennison et al., 2009). However, in this study, amphibian peptide (QUB 2841.38) did not show activity against *E. coli* after one hour of incubation at any concentrations tested. Low level of activity was observed after 3 to 4 hours of incubation at the highest concentrations tested (32 μg/ml). The possible reasonsfor such findings might be due to the selection of different antimicrobial assays and incubation times. In the present study, the amphibian peptide was incubated with *E. coli* in PBS for up to 4 hours; however, Dennison et al. (2009) carried out an overnight incubation of amphibian peptide with *E. coli*. Different from essential oils and other plant derived products, amphibian peptides might require longer incubation time to exert their antimicrobial effect. The reason for the inclusion of an amphibian peptide in this study was to investigate the possible synergistic effect of combining natural antimicrobial compounds and peptides as the latter has a different mode of antimicrobial action, e.g. boring pores at the cell membrane (Chen et al., 2003).

2.4.2 Evaluation of antibacterial activity against *Cl. sporogenes*

Polyphenols, green tea and apple E had the lowest MICs (0.57 and 0.68 μg/ml, respectively) against *Cl. sporogenes* among all the compounds tested. They were more active than the positive control antibiotic ampicillin. These compounds were also very effective against *E. coli*. Essential oils and their main components showed activities at concentrations ranging from relatively low to high when tested against *Cl. sporogenes*. The MIC of oregano oil was the lowest, 26 μg/ml, and its main constituent carvacrol was 59 μg/ml. Similar to the results obtained from *E. coli*, the difference in MIC between these two compounds might be due to other constituents in oregano oil which are also antibacterial and these components may exert their activity together. Onion oil also had a strong antibacterial effect against *Cl. sporogenes,* its MIC was 55 μg/ml, while cinnamon oil had a moderate effect (127 μg/ml) and citral displayed weak (472

μg/ml) activity. Only two of the organic acids, caffeic acid and chlorogenic acid, showed moderate and weak antibacterial activity against *Cl. sporogenes,* the MIC was 188 and 541 μg/ml respectively, while other organic acids did not show any activity even at the highest concentration tested.

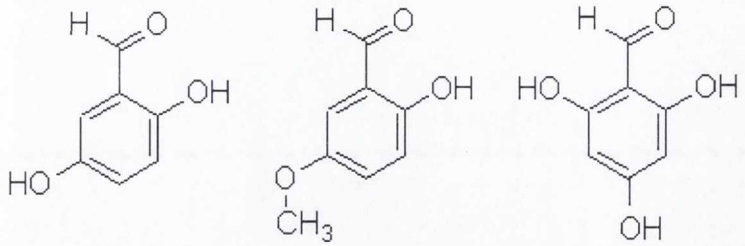
Different from the results obtained from *E. coli*, the benzaldehydes tested against *Cl. sporogenes* showed weak or no activity, while in a separate study (Wong et al., 2008), benzaldehydes showed strong antibacterial activity against Gram-positive bacterium, *Mycobacterium avium* subsp *paratuberculosis* (*Map*), which suggested that benzaldehyde might not be active against anaerobic bacteria. Cooking ingredients i.e. garlic and onion powder also showed no activity against *Cl. sporogenes* at the highest concentration tested, which was very different from the results obtained from *E. coli*, for which onion oil showed a relatively strong antibacterial activity (MIC was 55 μg/ml), the reason might due to the fact that the active components in garlic and onion powder are more effective against Gram-negative bacteria than Gram-positive bacteria. Another possibility is that the active components in onion oil are more readily available to *Cl. sporogenes* than the components present in the powdered form.

A large number of studies have demonstrated that naturally occurring compounds including cinnamaldehyde, carvacrol, oregano oil and green tea extract could significantly inhibit the spore germination of *Cl. perfringens* in different food models in a concentration dependent manner (Juneja et al., 2006, Juneja and Friedman, 2007, Juneja et al., 2007). In this study, green tea polyphenols showed significant antibacterial activity against *Cl. sporogenes*, while carvacrol and oregano oil were also active. However, cinnamaldehyde only exhibited an inhibitory effect at a relatively high concentration. *Cl. sporogenes* was used as a surrogate of *Cl. perfringens* in the present study.

Results of this study have shown that a number of naturally occurring compounds were active against *E. coli* K12 and *Cl. sporogenes*. Here several common methods have been used to determine the MIC of antibacterial compounds, including broth dilution method,

agar well and disc diffusion method. To facilitate an accurate comparison of the relative activities of naturally occurring compounds, we adopted the bactericidal assay developed by Friedman et al. (2002), as this method is considered to be more suitable for studying oil compounds due to their solubility issue. As oil compounds might not have a good diffusibility in agar medium, their effects therefore may not be effectively assessed. Moreover, incubation of the naturally occurring compounds with bacteria in PBS (not media) could help to evaluate the antibacterial properties of the compounds only and exclude any possible effects from the complex ingredients contained in the growth medium. Compared to the results of Friedman et al (2002), the compounds tested in this study have lower MICs. The possible reason may be that the non-pathogenic *E. coli* K12 used in this experiment might be more susceptible to antimicrobial compounds than the pathogenic strain *E. coli* O157:H7, which was used in their study.

The chemical structure and the position of the functional group of the natural compound might be important for their antibacterial activity. For instance, the three benzaldehydes tested showed different bactericidal activity against *E. coli*. 2,5-dihydroxybenzaldehyde exhibited the strongest activity, while 2,4,6-trihydroxybenzaldehyde and 2- hydroxyl-5-methoxybenzaldehyde showed moderate activity. The difference in terms of chemical structures between these compounds is merely the position of the functional group for 2,4,6-trihydroxybenzaldehyde and one different functional group for 2- hydroxyl-5-methoxybenzaldehyde (figure 2.13). It has been suggested that the possible modes of action of the naturally occurring compounds (e.g. essential oils) include disruption of cell membranes, inhibition of essential enzymes and chelation of essential trace elements such as iron and targeting the cell membranes (Friedman et al., 2002).



2,5-Dihydroxybenz-aldehyde

2-Hydroxy-5-methoxybenz-aldehyde

2,4,6-Trihydroxybenz-aldehyde

Figure 2.13 The structures of 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde and 2,4,6-trihydroxybenzaldehyde.

There is a long history of using essential oils as antimicrobials (Hili et al., 1997) and also in folk medicines. Carvacrol, the major component of widely consumed oregano oil, and *trans*-cinnamaldehyde, the major constituent of cinnamon oil, are considered as 'Generally Regarded as Safe (GRAS)' (Friedman et al., 2000, Burt, 2004, Adams et al., 2004). Antibiotics have been banned for incorporation into animal feedstuffs for disease prevention or growth promotion purpose. Therefore, the naturally occurring antibacterial compounds identified in this study may have the potential to be included in animal feed to replace the antimicrobial growth promoters for disease prevention or growth promotion. Further experiments could be performed, for example testing the bactericidal activity of naturally occurring compounds *in vivo* and their toxicity *in vitro*. Determining the modes of action of the naturally occurring compounds that showed strong bactericidal effects and performing synergistic trials to determine, if the naturally occurring compounds would exert a stronger antibacterial activity alone or in combination, which could help to maximize the potential of naturally occurring compounds.

To conclude, the antibacterial activities of naturally occurring compounds against *E. coli* K12 and *Clostridium sporogenes* were investigated. The most potent compounds against *E. coli* K12 was 2,5-dihydroxybenzaldehyde, followed by apple E polyphenols, gallic

acid, green tea polyphenols, garlic powder, onion powder and oregano oil. The most potent compounds against *Cl. sporogenes* were green tea polyphenols, followed by apple E polyphenols, oregano oil, onion oil and carvarcol. The benzaldehyde structure of the compound might be important for its antibacterial activity.

3 Antibacterial Activities of Naturally-Occurring Compounds against *Mycobacterium avium* subspecies *paratuberculosis*

3.1 Introduction

Mycobacterium avium subspecies *paratuberculosis* (*Map*) is the causative agent of Paratuberculosis, also known as Johne's disease, in wild and domestic ruminants, especially dairy cattle (Clarke, 1997, Wells and Wagner, 2000, Saxegaard, 1985, Hirst et al., 2004, Roussel et al., 2005, McKenna et al., 2006). *Map* may also have some roles in development of Crohn's disease and diabetes in humans (Naser et al., 2004, Hermon-Taylor et al., 1998, Scanu et al., 2007, Uzoigwe et al., 2007, Sechi et al., 2008). Johne's disease is a chronic granulomatous enteropathy (Berger et al., 2007), the clinical signs of which include chronic diarrhoea, progressive weight loss, decreased milk production and infertility (Cocito et al., 1994). There is currently no approved drug or treatment for Johne's disease (Parrish et al., 2004). Once farm animals are infected by *Map*, they are culled early (Rowe and Grant, 2006). The disease can lead to significant economic loss of livestock and dairy producers due to reduced productivity, animal wasting and mortalities in severe cases (Berger et al., 2007, Rowe and Grant, 2006).

Because Johne's disease occurs worldwide (Clarke, 1997), its global impact should not be under-estimated. Transmission of *Map* is through the faecal-oral route between animals (Parrish et al., 2004). Animals usually become infected with *Map* early in their lives as neonates when their immune system is not fully developed (Cocito et al., 1994). Before the onset of the clinical symptoms, there is a latent period that could last for 2 to 5 years. During the latent period the carrier animals could infect surrounding animals and the farm environment through the shedding of the *Map* into their faeces (Clarke, 1997). A farmer only realises the infection of their animals with *Map* when clinical signs are observed. At that stage the farm and other animals within the herd will have been already exposed to *Map* pathogen.

Milk could become contaminated with *Map* as it is systemic (Sweeney et al., 1992) or by faeces from infected animals during milking (Grant et al., 2002). Recent studies have shown that *Map* could survive pasteurisation (Grant et al., 1996, Grant et al., 1998, Grant et al., 1999, Grant et al., 2002, Grant et al., 2005), suggesting a possible risk to human health. Other attempts to inactivate *Map* include the use of UV irradiation (Altic et al., 2007), pulsed electric fields (Rowan et al., 2001) and hydrostatic pressure (López-Pedemonte et al., 2006). Another possible route of transmission of *Map* from cattle to humans is via contaminated water (Whan et al., 2001). This animal pathogen could be a hidden food safety threat (Stabel, 1998) that necessitates further study to help to prevent or control the spread of the organisms to either farm animals or humans.

Many antimicrobial compounds, mainly synthetic products, have been tested for their anti-*Map* effect (Parrish et al., 2004, Parrish et al., 2001, Williams et al., 1999, Chiodini, 1990, Zanetti et al., 2006, Brumbaugh et al., 2004). As antibiotics are associated with the development of antibiotic resistant bacteria (Phillips et al., 2004) that can pass to human via the food chain (World Health Organisation, 1996), their uses in food production are restricted and must be minimised (World Health Organisation, 2001).

Many naturally-occurring compounds, such as essential oils and phenolic compounds have been evaluated extensively for antimicrobial activity and chemically characterized (Friedman et al., 2006b, Friedman et al., 2004a, Friedman et al., 2002, Friedman et al., 2003, Friedman et al., 2004b). They are secondary plant metabolites and can be obtained naturally from different parts of plant materials of common herbs, spices and teas, including flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots by steam distillation, expression, fermentation or extraction (Prabuseenivasan et al., 2006) or may be synthesized. They are ideal for study as most of them are 'Generally Recognised As Safe' (Kabara, 1991). Most studies have focused on foodborne pathogens and food spoilage bacteria. However, some studies have involved *Mycobacterium* species such as the human pathogen *M. tuberculosis* (Figueroa et al., 2007, Gibbons et al., 2003) and other fast growing mycobacteria including *M. aurum*, *M. fortuitum*, *M. phlei*, *M. smegmatis* (Stavri et al., 2003, Schinkovitz et al., 2003) and *M. abscesus* (Schinkovitz et

al., 2003). Previous studies on mycobacteria have investigated the effect of crude plant extracts and their isolated constituents (Gutierrez-Lugo et al., 2005), from either the aerial part (Gutierrez-Lugo et al., 2005) or the root (Seidel and Taylor, 2004) of the plant, and some of these have shown promising results. To our knowledge, this study represents the first evaluation of the activity of naturally-occurring compounds against *Map*, an important animal pathogen and potential human pathogen. There is enormous scope for exploring these naturally-occurring compounds as potential nutraceuticals as replacements for discontinued standard antibiotics in feeds for food animals as well as potential food additives.

In this study, a total of 18 naturally occurring compounds including essential oils and some of their isolated constituents, apple and green tea polyphenols, and other plant extracts were screened for possible antimicrobial properties against three strains of *Mycobacterium avium* subspecies *paratuberculosis*: a bovine isolate, a raw-milk isolate, and a human isolate, using a macrobroth susceptibility method.

3.2 Materials and methods

3.2.1 Test compounds

The following compounds were originally obtained from Sigma (St. Louis, MO): 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, caffeic acid, capsaicin (natural), carvacrol, chlorogenic acid hemihydrates, *trans*-cinnamaldehyde, *trans*-cinnamic acid, citral (*cis* + *trans*), gallic acid, geraniol, methyl cinnamate and vanillic acid. The purity levels of these compounds ranged from 95 to 99.9% according to the manufacturer. Cinnamon oil (Cassia) was originally obtained from Yerba Buena Co. (Berkeley, Calif.). Oregano oil (Origanum) was originally obtained from Lhasa Karnak Herb Co. (Berkeley, Calif.). Their purity levels were not specified. Apple E (concentrated apple polyphenols) was originally obtained from Apple Poly LLC (Morrill, NE); the purity level was approximately 82%. Green tea polyphenols were originally obtained from LKT Laboratories, Inc. (St. Paul, MN); the purity level was not specified. The above compounds were gifts from Dr Mendel Friedma, Western Regional Research Center, U.S. Department of Agriculture, Albany, California, USA. Rifampicin (Sigma) was used as a positive control antibiotic as it is known to be active against *Map*.

3.2.2 Preparation of test compounds for susceptibility testing

A stock solution, 50 mg/ml for solid compounds and 50 µl/ml for oil compound, was prepared by suspending each test compound in absolute ethanol. Rifampicin was prepared as a 10 mg/ml stock suspension in absolute ethanol. The stock solutions were stored in aluminium foil-wrapped bottles at 4°C. As a high percentage of ethanol could be bactericidal, the amount of ethanol being added to the growth medium was kept as low as possible in order to minimize potential effect on the growth of *Map*. A preliminary experiment was carried out to determine the maximum percentage of ethanol which could be included in the growth medium without growth inhibition on *Map*. This was found to be 0.7% (vol/vol) (data not shown). The final concentration of ethanol present in the growth medium was standardized at 0.4% (vol/vol) in this study.

Before the test, each stock solution was serially diluted (3-fold) in absolute ethanol, 17.8 µl of each dilution was then added to sterile screw-cap test tubes (Sterilin, Barloworld Scientific, Staffordshire, U.K.) containing 3.9 ml of Middlebrook 7H9 broth to yield final concentrations of 74, 24.7, 8.2, 2.7 and 0.9 µg/ml for solid compounds and nl/ml for oil compounds, respectively. The concentrations of oil compounds will be expressed later as µg/ml according to the density of each oil compound. For rifampicin, the final concentrations were 10, 5, 2.5, 1.3 and 0.6 µg/ml. For apple E and green tea polyphenols, precipitations were observed after addition to the growth medium due to the presence of Tween 80. The complete growth medium used for testing these two compounds was therefore supplemented with 0.2% (vol/vol) of glycerol instead of 0.05% (wt/vol) Tween 80. Ethanol at 0.4% (vol/vol) was added to the growth medium to serve as negative controls.

3.2.3 *Mycobacterium avium subsp. paratuberculosis* strains

Three *Map* strains (ATCC 43015, NCTC 8578 and 806R) were tested in this study. ATCC 43015 is a human isolate obtained from the American Type Culture Collection. NCTC 8578 is a bovine isolate obtained from the National Collection of Type Cultures, Colindale, London. 806R was isolated from raw cow's milk by Grant et al. (Grant et al., 2002). The *Map* strains were prepared for testing as described by Whan et al. (Whan et al., 2001) with slight modifications. Briefly, the strains were maintained in Cryobank vials (Mast Group Ltd, Merseyside, U.K.) at -70°C. Two cryobeads were inoculated into 10 ml Middlebrook 7H9 broth (Difco Laboratories, Detroit, USA) supplemented with 0.05% (wt/vol) Tween 80 (Sigma), 10% (vol/vol) Middlebrook Oleic Albumin Dextrose Catalase (OADC) supplement (Difco), and 2 µg/ml of mycobactin J (Synbiotics Europe SAS, Lyon, France), pH 6.6 ± 0.2, and incubated at 37°C with gentle shaking (100 rpm) for 3 to 4 weeks until the optical density (OD) at 600nm (OD_{600}) was between 0.7 to 0.9.

3.2.4 Preparation of *Map* for susceptibility testing

The *Map* culture was de-clumped by adding 10 x 3 mm sterile glass beads and vortexing at high speed for 2 min, then rested for 2 min and repeated three times. The culture was transferred to a sterile test tube and the OD was determined using a cell density meter

(WPA Biowave CO8000) at 600 nm. OD was then adjusted to 0.10 by addition of Maximum Recovery Diluent (Oxoid, Unipath Ltd, Basingstoke, U.K.) to give a concentration of approximately 4.8 x 10^7 cfu/ml, which was later verified by diluting and plating the culture onto Herrold's Egg Yolk medium supplemented with 2 μg/ml mycobactin J (HEYM). The agar plates were sealed with Duraseal laboratory sealing film (Diversified Biotech, Boston, MA, U.S.A.) and incubated at 37°C for 4 to 6 weeks before colonies were counted.

3.2.5 Macrobroth susceptibility testing method

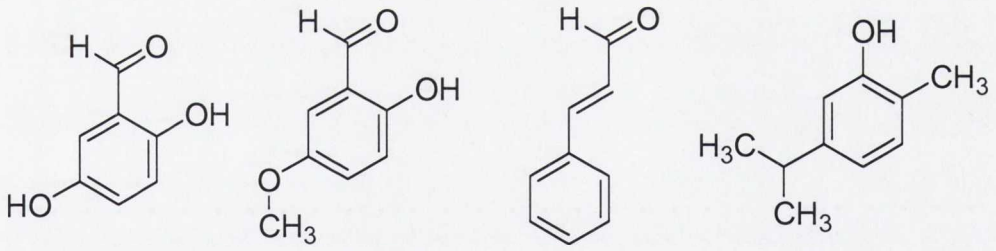
Sterile test tubes containing Middlebrook 7H9 broth with the test compounds added (as described above) were inoculated with 100 μl of the adjusted bacterial suspension. Thus the concentration of *Map* in each test tube was approximately 1.2 x 10^6 cfu/ml. The tubes were incubated at 37°C without shaking with caps screwed on tightly and OD_{600} was monitored at regular intervals for a period of 42 days. OD_{600} results were recorded and growth curves were plotted for all the test compounds at all concentrations in order to compare against the negative control culture. Some of the test compounds were coloured, which made reading OD_{600} impossible, so these test tubes were observed daily to check for the presence of culture settled at the bottom, if any, compared to the negative control. The entire experiment was repeated twice for the three *Map* strains.

3.2.6 Minimum Inhibitory Concentration (MIC) estimation

The lowest concentration of test compound in test tubes with no visible or detectable bacterial growth was considered to represent the minimum inhibitory concentration (MIC). For test tubes that showed bacterial growth, samples were taken from the tubes containing the highest concentration of test compounds that showed no inhibitory effect for acid-fast staining to confirm that the cultures had not become contaminated and were still pure *Map* cultures.

3.3 Results

Six of the 18 compounds tested were found to inhibit the growth of all three *Map* strains during incubation at 37°C for up to 42 days. The most effective compound was *trans*-cinnamaldehyde with the MIC at 25.9 μg/ml, followed by cinnamon oil at 26.2 μg/ml, oregano at 68.2 μg/ml, carvacrol at 72.2 μg/ml, 2,5-dihydroxybenzaldehyde at 74 μg/ml and 2-hydroxy-5-methoxybenzaldehyde at 90.4 μg/ml (table 3.1). Three of these compounds were aldehydes (*trans*-cinnamaldehyde, 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde (figure 3.1)). *Trans*-cinnamaldehyde is the main active ingredient (81%) in cinnamon cassia oil (Friedman et al., 2002). The phenolic compound carvacrol (figure 3.1) is the major component in oregano oil (Veldhuizen et al., 2007). These results suggest that the aldehyde group of the three compounds, the phenolic group of carvacrol and/or its hydrophobic-hydrophilic nature may be important for antimicrobial activity. The MICs of these six compounds were the same for all three strains of *Map*. The chemical structures of *trans*-cinnamaldehyde, 2-hydroxy-5-methoxybenzaldehyde, 2,5-dihydroxybenzaldehyde and carvacrol show slight similarity to isoniazid and pyrazinamide, drugs shown to be effective against *M. tuberculosis* (Sun et al., 1997).



2,5-Dihydroxybenz-aldehyde

2-Hydroxy-5-methoxybenz-aldehyde

trans-cinnamaldehyde

carvacrol

Figure 3.1 The structures of 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, *trans*-cinnamaldehyde and carvacrol.

Table 3.1 Minimum Inhibitory Concentration (MIC) (μg/ml) of naturally-occurring compounds tested for activity against three *Map* strains.

Test compound	MIC (μg/ml) for:		
	NCTC 8578	ATCC 43015	806R
Rifampicin	<0.6[a]	<0.6	<0.6
2,5-dihydroxybenzaldehyde	74	74	74
2-hydroxy-5-methoxybenzaldehyde	90.4	90.4	90.4
Carvacrol	72.2	72.2	72.2
Cinnamon oil (Cassia)	26.2	26.2	26.2
Oregano oil (Origanum)	68.2	68.2	68.2
Trans-Cinnamaldehyde	25.9	25.9	25.9
2,4,6-trihydroxybenzaldehyde	-[b]	-	-
Apple E (apple polyphenols)	-	-	-
Caffeic acid	-	-	-
Capsaicin (natural)	-	-	-
Chlorogenic acid hemihydrates	-	-	-
Citral (*cis* + *trans*)	-	-	-
Gallic acid	-	-	-
Geraniol	-	-	-
Green tea polyphenols	-	-	-
Methyl cinnamate	-	-	-
Trans-Cinnamic acid	-	-	-
Vanillic acid	-	-	-

[a] Growth of *Map* was inhibited by the lowest concentration of Rifampicin tested.

[b] Growth of *Map* was not inhibited in the presence of the highest concentration of compound tested.

The macrobroth susceptibility testing method adopted for this study essentially mimics the BACTEC 12B or MGIT culture approach for antibiotic susceptibility testing of mycobacteria (Zanetti et al., 2006). The macrobroth method was used because we had no access to the BACTEC 460 or MGIT 960 instruments. A growth curve for *Map* was plotted for each test compound at different concentrations based on the OD_{600} values, in order to compare differences in growth characteristics, i.e. length of lag phase and maximum OD_{600} achieved by the culture.

The growth curves of *Map* NCTC 8578 in Middlebrook 7H9 broth with the addition of *trans*-cinnamaldehyde at different concentrations and negative control of 0.4% (vol/vol) ethanol are shown in figure 3.2. This figure shows a concentration-dependent inhibitory effect of *trans*-cinnamaldehyde on the growth of *Map* NCTC 8578, with the complete inhibition of growth at and above the minimum inhibitory concentration of 25.9 µg/ml.

The growth curves of *Map* ATCC 43015 and 806R in Middlebrook 7H9 broth with the addition of 2,5-dihydroxybenzaldehyde and carvacrol at different concentrations and negative control of 0.4% (vol/vol) ethanol are shown in figures 3.3 and 3.4 respectively. These figures show a concentration-dependent inhibitory effect of 2,5-dihydroxybenzaldehyde and carvacrol on the growth of *Map* ATCC 43015 and 806R, with the complete inhibition of growth at the minimum inhibitory concentrations of 74 µg/ml and 72.2 µg/ml.

Figure 3.5 illustrates the growth of *Map* NCTC 8578 in Middlebrook 7H9 broth supplemented with minimum inhibitory concentrations of *trans*-cinnamaldehyde (25.9 µg/ml), cinnamon oil (26.2 µg/ml), oregano oil (68.2 µg/ml), carvacrol (72.2 µg/ml), 2,5-dihydroxybenzaldehyde (74 µg/ml), 2-hydroxy-5-methoxybenzaldehyde (90.4 µg/ml). The non-inhibitory concentrations of ethanol (0.4% vol/vol, negative control), vanillic acid (74 µg/ml) and citral (65.7 µg/ml) are also shown in figure 3.5. This figure illustrates the complete inhibition of *Map* growth by the six inhibitory compounds, whereas growth of *Map* was not inhibited by vanillic acid and citral at the highest concentrations tested or by the negative control (0.4% ethanol).

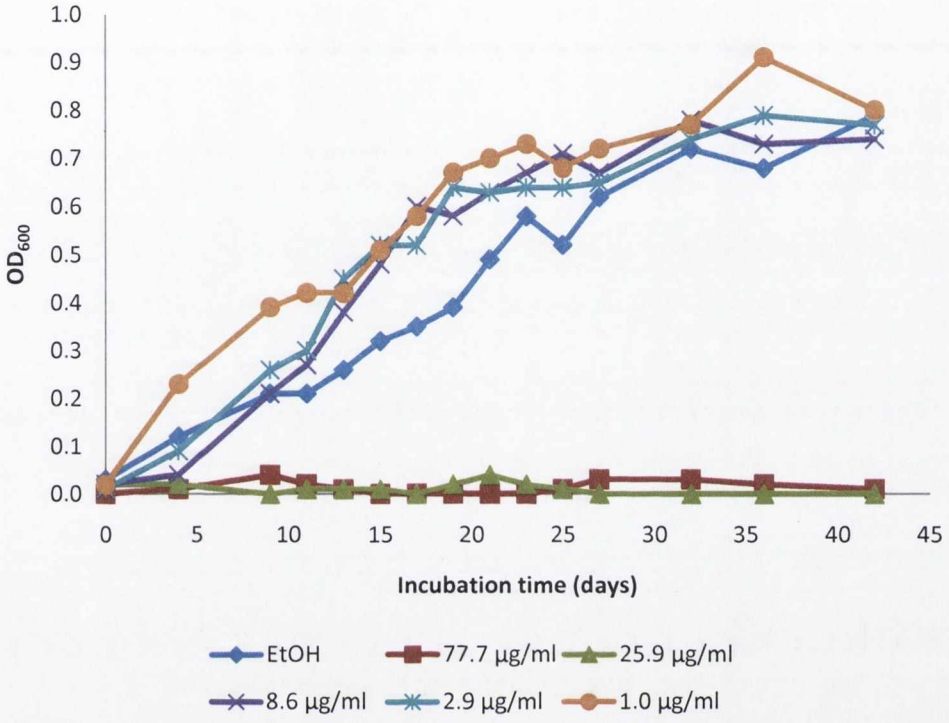


Figure 3.2 Effect of different concentrations (µg/ml) of *trans*-cinnamaldehyde and non-inhibitory concentrations of ethanol (0.4%, negative control) on the growth of *Map* NCTC 8578 in Middlebrook 7H9 broth.

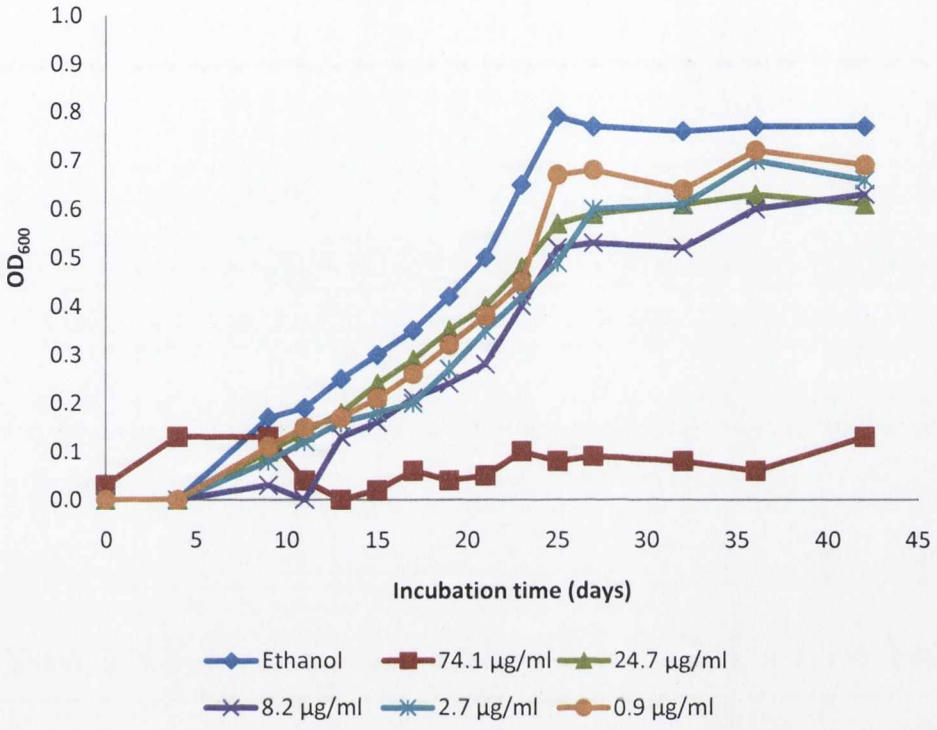


Figure 3.3 Effect of different concentrations (µg/ml) of 2,5-dihydroxybenzaldehyde and non-inhibitory concentrations of ethanol (0.4%, negative control) on the growth of *Map* ATCC 43015 in Middlebrook 7H9 broth.

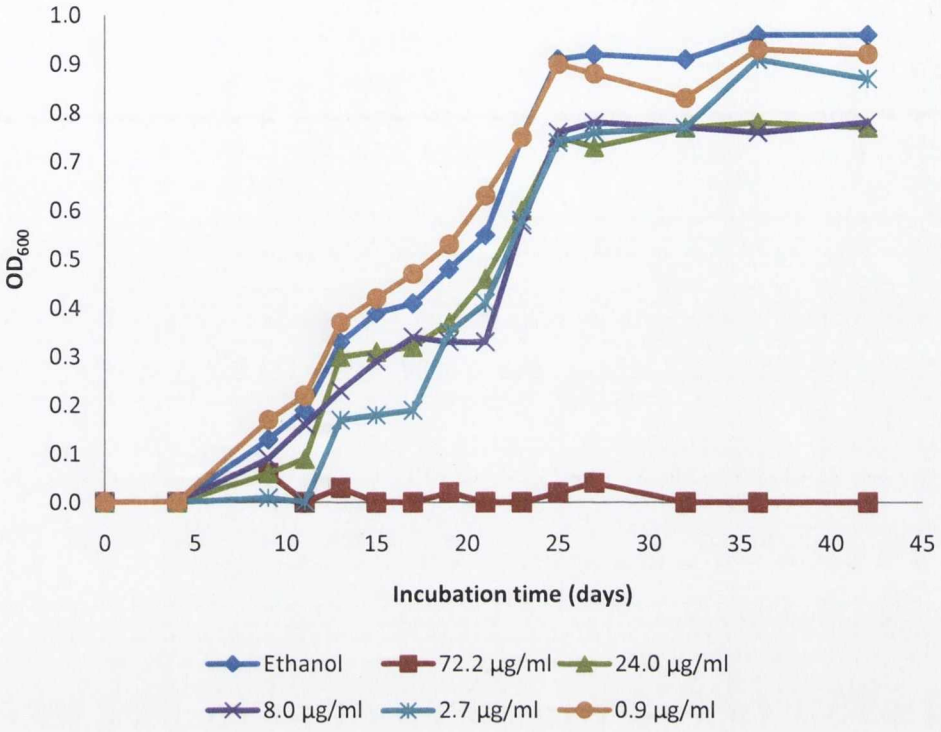


Figure 3.4 Effect of different concentrations (µg/ml) of carvacrol and non-inhibitory concentrations of ethanol (0.4%, negative control) on the growth of *Map* 806R in Middlebrook 7H9 broth.

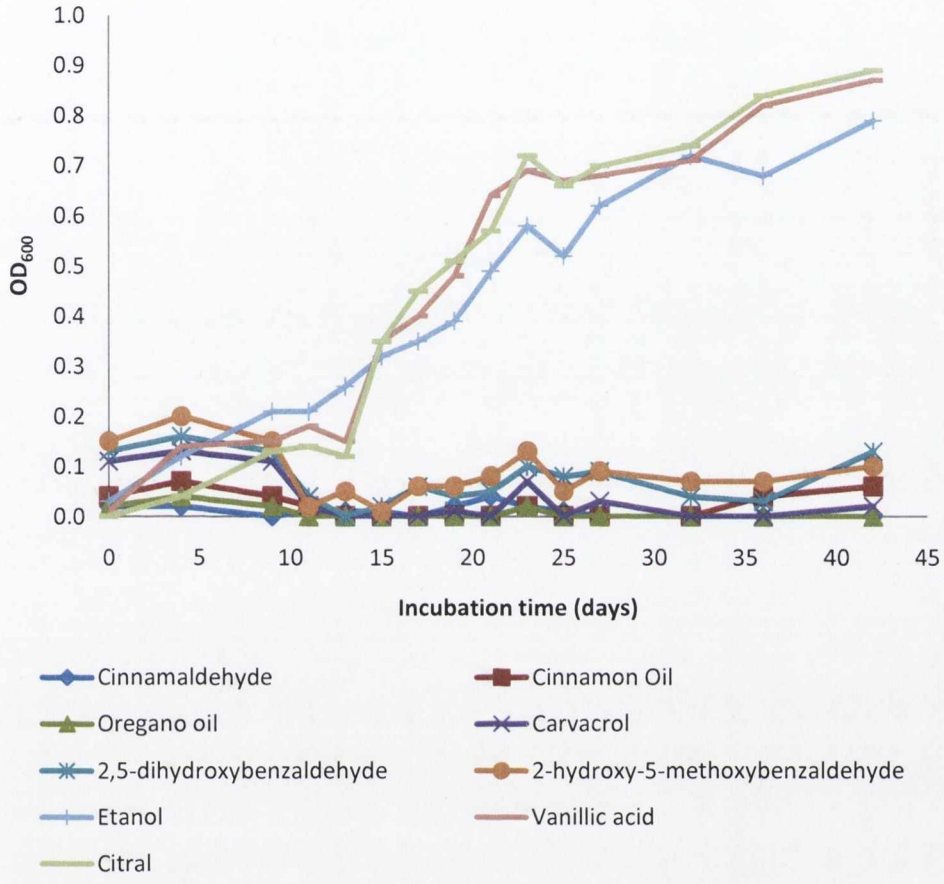


Figure 3.5 Growth of *Map* NCTC 8578 in Middlebrook 7H9 broth supplemented with the six active compounds at their MICs: *trans*-cinnamaldehyde (25.9 µg/ml), cinnamon oil (26.2 µg/ml), oregano oil (68.2 µg/ml), carvacrol (72.2 µg/ml), 2,5-dihydroxybenzaldehyde (74 µg/ml) and 2-hydroxy-5-methoxybenzaldehyde (90.4 µg/ml), ethanol (0.4%, negative control), vanillic acid (74 µg/ml) and citral (65.7 µg/ml). 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde had slightly higher initial OD values as they produced coloured solutions.

3.4 Discussion

There is a long history of using essential oils as antimicrobials beginning as early as 1910 (Hili et al., 1997). According to a recent series of studies carried out by Friedman and colleagues, five out of the six compounds that were found to be active against *Map* in this study - cinnamon oil, *trans*-cinnamaldehyde, oregano oil, 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde - have been shown to inhibit the growth of foodborne pathogens such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enterica* (Friedman et al., 2002, Friedman et al., 2003), whilst - carvacrol, cinnamon oil and oregano oil - were also found to be active against antibiotic-resistant *Staphylococcus aureus* and *Bacillus cereus* vegetative cells and spores as well as against resistant *Micrococcus luteus* (Friedman et al., 2004a, Friedman et al., 2006a).

The published MICs for the five active compounds, expressed as reduction of 50% of colony forming units (cfu), against *Campylobacter jejuni* ranged from 6.6 to 190 μg/ml, 430 to 1,100 μg/ml for *Escherichia coli* O157:H7, 80 to 1,900 μg/ml for *Listeria monocytogenes* and 330 to 1,900 μg/ml for *Salmonella enterica* (Friedman et al., 2002, Friedman et al., 2003). In a separate study (Friedman et al., 2004a), the antimicrobial activities of test compounds carvacrol, cinnamon oil and oregano oil were expressed as the cfu recovered from the bactericidal assay tested with different compounds compared to the cfu recovered from the negative control.

Adopting the same definition of MIC from the previous studies, i.e. a 50% of CFU reduction or more (as some compounds either showed no inhibition or inhibition over 50% of the cfu at the next higher concentration), the MICs of carvacrol, cinnamon oil and oregano oil against *Staphylococcus aureus* was 66.7 μg/ml or above, *Bacillus cereus* vegetative cells ranged from 0.0667 to 66.7 μg/ml. No inhibition was observed against *Bacillus cereus* spores. When our findings are compared with those of the previously described studies, one similarity which is apparent is that the MICs of the six compounds that were found to be active against *Map* fell within the same range as for the other foodborne pathogens studied. However, due to the slow-growing nature of

Map and its fastidious nutritional requirements, the assay methods and way of determining the MICs in this study are different from the methods used by Friedman et al. in (Friedman et al., 2004a, Friedman et al., 2006b, Friedman et al., 2002, Friedman et al., 2003, Friedman et al., 2004b). It is therefore difficult to directly compare the effectiveness of the six compounds against *Map* and the other pathogens mentioned above.

The mechanism of antimicrobial activity of naturally occurring compounds is thought to be of a specific nature rather than non-specific since it is concentration dependent (Friedman et al., 2002). Possible modes of action of the essential oils include disruption of cell membranes, inhibition of essential enzymes and chelation of essential trace elements such as iron and targeting the cell membranes (Friedman et al., 2002). Ultee et al. (1999) found that after treating *B. cereus*, a gram-positive bacterium, with carvacrol, the permeability of the cell membrane was increased for essential ions like potassium ions and hydrogen ions, consequently causing leakage of essential ions out of the cells. As a result of this leakage, enzymes might not be able to function properly which could affect the turgor pressure, DNA synthesis and other metabolic activities. They also found that after the treatment with carvacrol, the ATP concentration inside the cells declined but that there was no leakage of ATP to the external environment. It is possible that carvacrol might have reduced the rate of ATP synthesis or increased ATP hydrolysis.

In a related study, Helander et al. (1998) also found that carvacrol exerted an effect on cell membranes. They observed leakage of cellular material but also of ATP. These results differ from the finding of Ultee et al. (1999). This difference may be due to the fact that Helander et al. (1998) used gram-negative bacteria. Lambert et al. (2001) found that besides leakage of potassium and phosphate ions, changes in the internal pH of the treated cells also occurred. There was a reduction of pH gradient across the cytoplasmic membrane, affecting pH homeostasis.

Currently there is no drug approved to treat Johne's disease, the anti-*Map* naturally-occurring compounds identified in this study may have the potential to be included in therapeutic drugs for the treatment of Johne's disease in farm animals, and possibly also in human medicine against Crohn's disease. Carvacrol, the major component of the widely consumed oregano oil, and *trans*-cinnamaldehyde, the major constituent of cinnamon oil, are already designated as generally regarded as safe (Friedman et al., 2000, Burt, 2004, Adams et al., 2004). However, we have no information about the safety of 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde. Further research will focus on the mode of action of the six effective compounds against *Map* and toxicity testing will be performed in order to confirm the potential of these compounds to be used as additives in animal feedstuffs and human foods.

As *Map* is suggested to be present in milk and other dairy products and certain strains of *Map* could be resistant to acidic environments (Sung and Collins, 2003). A further study was designed to determine if the 6 anti-*Map* compounds would still exert their antibacterial effects in acidic environment. However, in the preliminary pH challenge test, we found that *Map* NCTC 8578 was not able to grow in Middlebrook 7H9 broth after the pH was adjusted to acidic values using acetate acid (figure 3.6). Therefore, no further study of antibacterial activities of 6 anti-Map compounds at low pH was carried out.

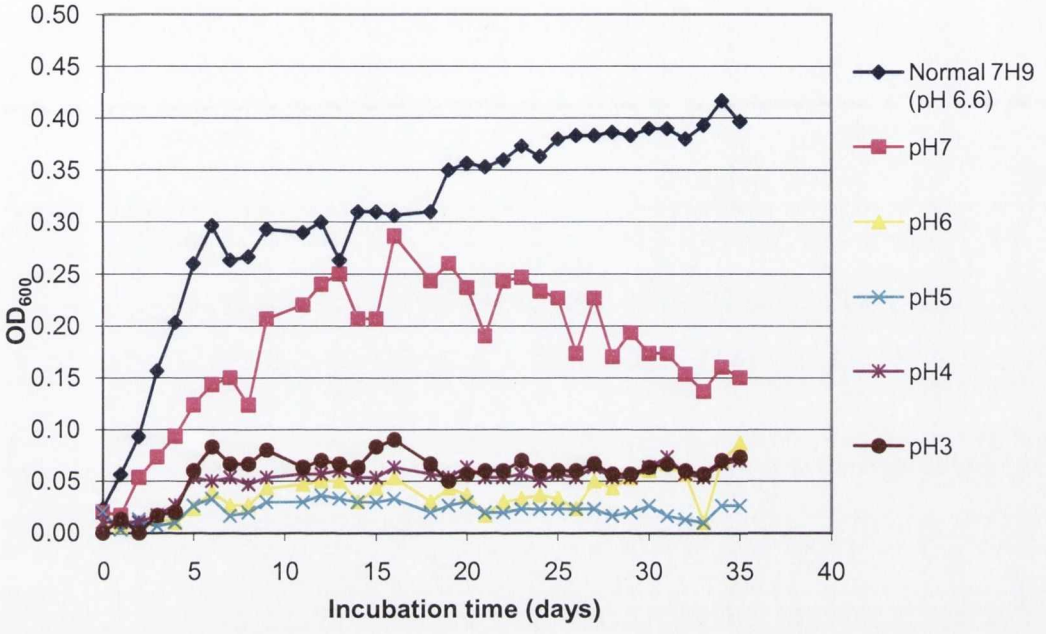


Figure 3.6 Effect of different pH on the growth of *Map* NCTC 8578 in Middlebrook 7H9 broth.

4 Synergistic study of antimicrobial activities of naturally-occurring compounds against *Mycobacterium avium* subspecies *paratuberculosis*

4.1 Introduction

Research on the application of naturally occurring compounds has drawn much attention in agricultural food industries both in food preservation and animal production, as consumers increasingly demand natural quality and chemical-free foods. Increasing scientific evidence also suggests the prominent antimicrobial effects of the naturally occurring compounds can make them ideal as a substitute for chemical preservatives (Ultee, 1998, Friedman et al., 2002). Apart from extending the shelf life of food products, there is also a huge amount of interest in exploring the possible application of naturally occurring compounds as feed additives for food animal production, in order to maintain the health of the animal, control outbreak of diseases, to decrease mortality rate and to promote the growth.

Naturally occurring compounds from both plant and animal origins are both popular in modern research, as they are unlikely to enhance the development of antimicrobial resistance. Antimicrobials of plant origin have shown prominent effect against different human and animal pathogens, especially those causing foodborne diseases, e.g. *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* in buffer (Friedman et al., 2002), culture media (Ultee, 1998) and food models (Fisher and Phillips, 2006). Antimicrobial peptides from amphibians, e.g. Aurein 2.5, had shown prominent effects on both planktonic solutions and bacterial biofilms of pathogenic *Bacillus subtilis* and non-pathogenic *Escherichia coli*, it was suggested that it may be used for decontamination of medical devices (Dennison et al., 2009). However, there are drawbacks of using plant products such as essential oils and their components and antimicrobial peptides in the food industry. Firstly, these compounds have a distinctive smell, taste and colour of the essential oils and their components. Secondly is

the possible cytolytic and haemolytic activity of antimicrobial peptides (Conlon et al., 2009a, Conlon et al., 2009b).

Synergistic effect refers to the interaction of two or more compounds, e.g. drugs, when combined, would produce a total effect that is greater than the sum of the individual compounds. Synergistic study is important and is also a common tool in many life science areas especially in medical and health research for pharmaceutical drug evaluation and development. Many studies have been carried out on combinations of different antibiotics (Miranda-Novales et al., 2006), combinations of antibiotics and natural antimicrobials (Shahverdi et al., 2007, Hemaiswarya and Doble, 2009, Kim et al., 2009), combinations of different natural antimicrobials (Nazer et al., 2005) and combinations of different natural antimicrobials and physical treatments, e.g. heat and refrigeration (Valero and Francés, 2006).

The aims of these studies were to investigate the effects of a combination of different antimicrobial compounds, at lower concentrations, on their antimicrobial activities compared to using the compounds alone. If synergitic effects exist in combinations, they could be applied at lower concentrations which could overcome some of the problems of using a natural product in the food industry. When essential oils or their components are used at low quantities in food products, the natural quality or the sensory acceptance of the food products might be maintained and when a low quantity of antimicrobial peptides is used, the risk of cytolytic or haemolytic effects might be minimised. It might also help to reduce the cost of an effective therapeutic formula.

The two bacteria selected in this study are pathogens of farm animals, *Map* and its surrogate *M. smegmatis* MC^2155. *Map* is a notorious animal pathogen causing Johne's disease in cattle, which causes huge economic loss and may play a role in human Crohn's disease. Due to the very slow growth rate of *Map*, a surrogate *M. smegmatis* MC^2155 was later introduced into the study. It is a faster growing mycobacterium. Two amphibian peptides and the six identified anti-*Map* natural compounds originating from plants were subjected to the present study and their antimicrobial effects on *Map* were

examined. Two susceptibility assays were used in this study, which were macrobroth dilution assay and microbroth dilution assay. Macrobroth dilution assay was performed in test tubes, the optical density at 600 nm (OD_{600}) was measured at regular intervals to indicate the increase of cell density, i.e. growth of cell population, in the test tube over time. Due to the relatively large volume (minimum 3 ml) of culture medium and test compounds and long incubation time (42 days for *Map*) were required in the macrobroth dilution assay, microbroth dilution assay was later adopted in our study. Microbroth dilution assay was performed in microtiter plates, only a relatively small volume (200 μl) of a relatively young culture (14 days) was required for each test well, metabolic indicator alamarBlue® reagent was added for colour development, which is in proportion to the degree of metabolic activity. We also studied the possible synergistic antimicrobial effects of the six anti-*Map* compounds and a herbal extract of *Coptis chinensis Franch* (Aw) on *M. smegmatis* MC^2155.

4.2 Materials and methods

4.2.1 Test compounds

The following compounds were originally obtained from Sigma (St. Louis, MO): 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, carvacrol and *trans*-cinnamaldehyde. The purity levels of these compounds ranged from 95 to 99.9% according to the manufacturer. Cinnamon oil (Cassia) was originally obtained from Yerba Buena Co. (Berkeley, Calif.). Oregano oil (Origanum) was originally obtained from Lhasa Karnak Herb Co. (Berkeley, Calif.). Their purity levels were not specified. The above compounds were gifts from Dr Mendel Friedma, Western Regional Research Center, U.S. Department of Agriculture, Albany, California, USA. Rifampicin (Sigma) was used as a positive control antibiotic as it is known to be active against *Map*. An aqueous extract of *Coptis chinensis Franch* (Aw) was obtained from the Institute of Agri-Food and Land Use, School of Biological Sciences, Queen's University Belfast. Amphibian peptides QUB 2036.44 and QUB 2841.38 from frog's origin; were obtained from the School of Pharmacy, Queen's University Belfast.

4.2.2 Preparation of test compounds

For those commercially available compounds, a stock solution, (50 mg/ml for solid compound and 50 µl/ml for oil compound) was prepared by suspending each test compound in absolute ethanol. Rifampicin was prepared as a 1 mg/ml stock suspension in absolute ethanol. The stock solutions were stored in aluminium foil-wrapped bottles at 4°C. The final concentration of ethanol present in the growth medium was standardized at 0.4% (vol/vol) in this study, as previous studies had shown that this percentage showed no growth inhibition on *Map* or *M. smegmatis* MC^2155 (data not shown). Amphibian peptides QUB 2036.44 and QUB 2841.38 were suspended in analytical grade DMSO (Sigma, UK) and then diluted in 50 mM PBS, the final peptide concentration was 2 mg/ml and the final DMSO concentration in the peptide stock was 10% (vol/vol). Herbal extract was suspended in DMSO (Sigma, UK) and then diluted in 50 mM PBS, the final concentration was 10 mg/ml and the final DMSO concentration in the Aw stock was 5% (vol/vol).

4.2.3 Bacterial strains and growth condition

Map strain NCTC 8578, a bovine isolate obtained from the National Collection of Type Cultures, Colindale, London, was tested in this study. The strain was maintained and grown as described in Chapter 3 of the thesis.

M. smegmatis MC^2155 was maintained and grown in similar conditions as *Map* except Tween 80 and mycobactin J were omitted in the growth medium but 2 mM $CaCl_2$ was added. $CaCl_2$ was prepared as a 500 mM stock solution, for every 10 ml of growth medium for *M. smegmatis* MC^2155, 40 µl of the stock was added to achieve the final concentration 2 mM. The culture was incubated at 37°C with gentle shaking (100 rpm) for 3 to 4 days until late log phase, i.e. the culture was still at a rapid growth rate but the nutrients in the growth medium were nearly depleted and further growth of the culture would soon be limited, was reached.

4.2.4 Preparation of strains for susceptibility testing

The *Map* and *M. smegmatis* cultures were de-clumped and prepared as described in Chapter 3 of the thesis.

For *M. smegmatis*, the OD was adjusted to 0.14 by addition of the culture medium to give a concentration of approximately 4.8×10^7 cfu/ml, which was later verified by diluting and plating the culture onto Middlebrook 7H10 agar (Difco Laboratories, Detroit, USA) supplemented with 0.02% (vol/vol) Glycerol (Sigma, UK) and 10% (vol/vol) Middlebrook Oleic Albumin Dextrose Catalase (OADC) supplement (Difco), pH 6.6 ± 0.2. The agar plates were sealed with Duraseal laboratory sealing film (Diversified Biotech, Boston, MA, U.S.A.) and incubated at 37°C for 3 to 4 days before *M. smegmatis* colonies were counted and 4 to 6 weeks before *Map* colonies were counted.

4.2.5 Combinations of amphibian peptides and naturally occurring compounds against *Map* using macrobroth susceptibility testing method

Sterile test tubes containing 3 ml of Middlebrook 7H9 broth medium with supplements as described above were added with 13.3 µl of naturally occurring compound stocks diluted in absolute ethanol to yield concentrations equivalent to one-third of their MICs (cinnamon oil: 8 µg/ml, *trans*-cinnamaldehyde: 8 µg/ml, oregano oil: 24 µg/ml, carvacrol: 24 µg/ml, 2,5-dihydroxybenzaldehyde: 24 µg/ml and 2-hydroxy-5-methoxybenzaldehyde: 24 µg/ml) and then 30 µl of amphibian peptide stocks diluted in 50 mM PBS, pH 7.0. Each naturally occurring compound was tested in combination with each amphibian peptide at three concentrations (5, 10 and 20 µg/ml). Ethanol at 0.4% (vol/vol) and DMSO at 0.1% (vol/vol) were added to the growth medium to serve as negative controls. Rifamipicin (0.6 µg/ml) was used as the positive control. Naturally occurring compounds at MICs (cinnamon oil: 24 µg/ml, *trans*-cinnamaldehyde: 24 µg/ml, oregano oil: 74 µg/ml, carvacrol: 74 µg/ml, 2,5-dihydroxybenzaldehyde: 74 µg/ml and 2-hydroxy-5-methoxybenzaldehyde: 74 µg/ml) on their own served as supplementary positive controls, while naturally occurring compounds at one-third of MICs on their own acted as supplementary negative controls. The test tubes were inoculated with 75 µl of the adjusted bacterial suspension. Thus the concentration of *Map* in each test tube was approximately 1.2×10^6 cfu/ml. The tubes were vortexed vigorously, incubated at 37°C without shaking with caps screwed on tightly and OD_{600} was monitored at regular intervals for a period of 42 days. OD_{600} readings, which represented the increase of bacterial population in terms of optical density, were recorded and growth curves were plotted in excels worksheet for comparison of the growth of culture. This experiment was carried out once only due to the relatively large volume (minimum 3 ml) of culture medium and test compounds and long incubation time (42 days) required. As the supply of amphibian peptides was limited; microbroth dilution susceptibility testing was later introduced into our study because smaller amounts of amphibian peptides, culture medium and shorter incubation time were required to perform the test.

4.2.6 Combinations of amphibian peptides and naturally occurring compounds against *Map* using microbroth susceptibility testing method

Sterile small bottles containing 1 ml of Middlebrook 7H9 broth medium with supplements as described above were added with 4.4 µl of naturally occurring compound stocks diluted in absolute ethanol to yield concentrations equivalent to one-third of their MICs (cinnamon oil: 8 µg/ml, *trans*-cinnamaldehyde: 8 µg/ml, oregano oil: 24 µg/ml, carvacrol: 24 µg/ml, 2,5-dihydroxybenzaldehyde: 24 µg/ml and 2-hydroxy-5-methoxybenzaldehyde: 24 µg/ml) and then 10 µl of amphibian peptide stocks diluted in 50 mM PBS, pH 7.0. Each naturally occurring compound was tested in combination with each amphibian peptide at three concentrations (5, 10 and 20 µg/ml). Ethanol at 0.4% (vol/vol) and DMSO at 0.1% (vol/vol) were added to the growth medium to serve as negative controls. Rifampicin (0.3, 0.6 and 1.2 µg/ml) served as positive control. Supplementary positive and negatives controls were set up as described previously. The small bottles were inoculated with 25 µl of the adjusted bacterial suspension. Thus the concentration of *Map* in each bottle was approximately 1.2 x 10^6 cfu/ml. The small bottles were vortexed vigorously, incubated at 37°C without shaking with caps screwed on tightly for 14 days. Two hundred microliters of samples were pipetted from each bottle into a sterile clear polystyrene 96-well plate in triplicate, added with 20 µl of alamarBlue® reagent (AbD Serotec, UK), which is a redox/metabolic indicator; and mixed gently. Plates were covered and sealed with parafilm and incubated at 37°C for colour development. Absorbance readings at 570 and 600 nm were then taken after 48 h. The assay was repeated twice. Percentage reduction values of alamarBlue (an indicator of metabolic activity) were determined using the formula provided by the manufacturer. According to the manufacturer, when cells are metabolizing they maintain a reducing environment within their cytosol and this reduced state can be measured spectrophotometrically through the conversion of colorimetric redox indicators, which is in proportion to the degree of metabolic activity; the higher the metabolism, the higher the reduced state. The microbroth dilution method was adopted as only a relatively small volume (200 µl) of a relatively young culture (14 days) was required for reduced state/metabolic activity measurement, which saved test materials and time compared to the macrobroth dilution assay.

4.2.7 Combinations of herbal extract *Coptis chinensis Franch* (Aw) and naturally occurring compounds against *M. smegmatis* MC^2155 using macrobroth susceptibility testing method

Sterile test tubes containing 3 ml of Middlebrook 7H9 broth supplemented with 2 mM $CaCl_2$ and 10% (vol/vol) OADC were added with naturally occurring compounds at half of their MICs. (*trans*-cinnamaldehyde: 50 µg/ml, oregano oil: 74 µg/ml, carvacrol: 74 µg/ml, 2,5-dihydroxybenzaldehyde: 148 µg/ml and 2-hydroxy-5-methoxybenzaldehyde: 148 µg/ml) and then 30 µl of herbal extract Aw. Ethanol at 0.6% (vol/vol) and DMSO at 0.05% (vol/vol) were added to the growth medium to serve as negative controls. Rifamipicin (148 µg/ml) served as positive control. Supplementary positive and negatives controls were set up using the MICs and one-third of MICs of each test compound respectively. The test tubes were inoculated with 75 µl of the adjusted bacterial suspension. Thus the concentration of *M. smegmatis* MC^2155 in each test tube was approximately 1.2×10^6 cfu/ml. The tubes were vortexed vigorously, incubated at 37°C without shaking with caps screwed on tightly and OD_{600} was monitored at regular intervals for a period of 7 days. OD_{600} results were recorded and growth curves were plotted in excels worksheet for comparison of the growth of culture. The experiment was repeated twice. Summary of the different combinations of natural products for synergistic studies in this study is shown in table 1.

4.2.8 Minimum Inhibitory Concentration (MIC) estimation.

Adopting the definition of MIC in the previous susceptibility testing on *Map*, the lowest concentration of test compound in test tubes or test wells with no visible or detectable bacterial growth was considered to represent the minimum inhibitory concentration (MIC).

Table 4.1 Combinations of test compounds against *Map* and *M. smegmatis* and susceptibility testing methods

Test compounds	QUB 2036.44	QUB 2841.38	Aw
trans-Cinnamaldehyde	*Map* (Macrobroth dilution and microbroth dilution susceptibility testing)	*Map* (Macrobroth dilution and microbroth dilution susceptibility testing)	*M. smegmatis* (Macrobroth dilution susceptibility testing)
Cinnamon oil			
Carvacrol			
Oregano oil			
2,5-dihydroxybenzaldehyde			
2-hydroxy-5-methoxybenzaldehyde			

4.3 Results

4.3.1 Combination of amphibian peptides and naturally occurring compounds against *Map* using macrobroth susceptibility testing method

As shown in figures 4.1.a and 4.1.b, in the presence of amphibian peptides QUB 284138 and QUB 203644 alone, the growth of *Map* was not inhibited, compared to the negative control, ethanol (0.4% vol/vol) and supplementary negative control, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde at one-third of their MIC (24 μg/ml), the growth of *Map* was visible earlier and the final OD achieved was not that high. However, when supplementing 5 μg/ml of non active amphibian peptides QUB 284138 and QUB 203644 with anti-*Map* compound 2,5-dihydroxybenzaldehyde at one-third of its MIC and 10 μg/ml of these two peptides with anti-*Map* compound 2-hydroxy-5-methoxybenzaldehyde at one-third of its MIC, it was found that the growth of *Map* was inhibited during incubation at 37°C for up to 42 days. This might have resulted from the synergistic effect of combining amphibian peptides and naturally occurring compounds together.

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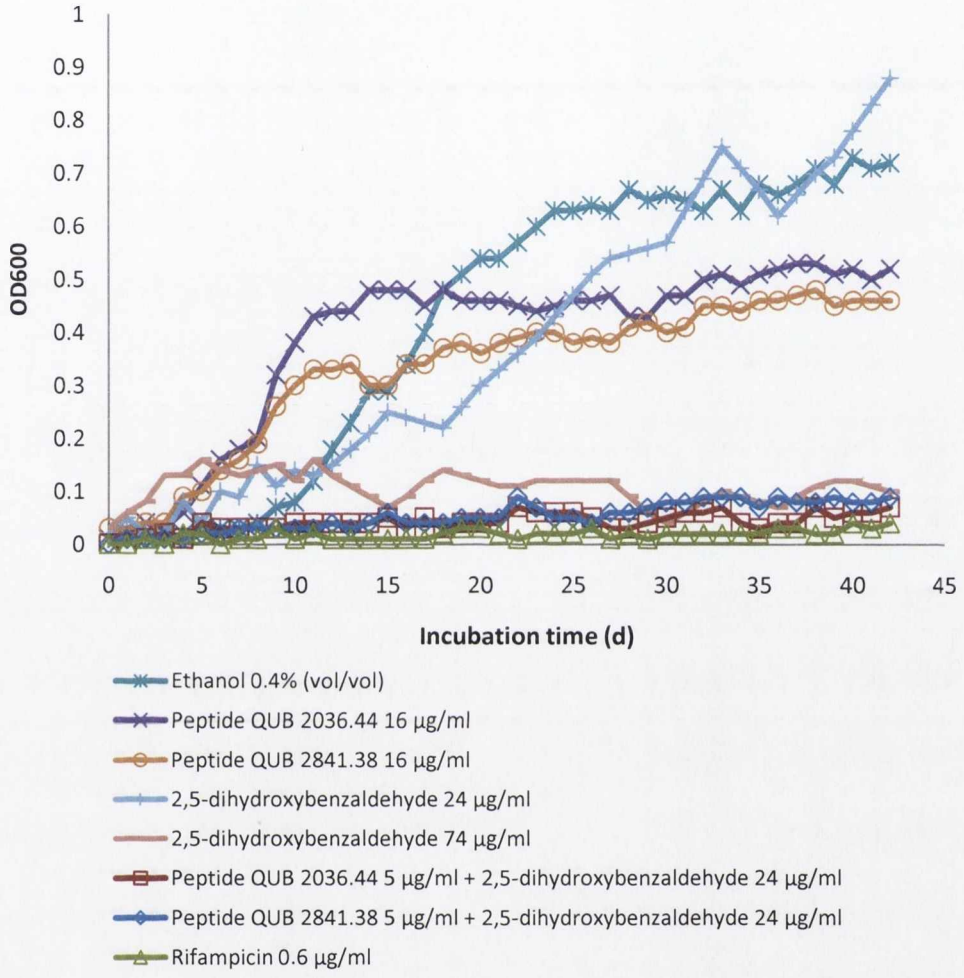

Figure 4.1.a Growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with the active compound 2,5-dihydroxybenzaldehyde at its MIC (74 µg/ml), one-third of its MIC (25 µg/ml), one-third of its MIC combined with non active peptide QUB 2036.44 (5 µg/ml) and QUB 2841.38 (5 µg/ml) respectively, QUB 2036.44 (16 µg/ml) and QUB 2841.38 (16 µg/ml) on their own, ethanol (0.4% vol/vol) (negative control) and rifampicin (0.6 µg/ml) (positive control). 2,5-dihydroxybenzaldehyde shows slightly higher initial OD values as it produced coloured solutions.

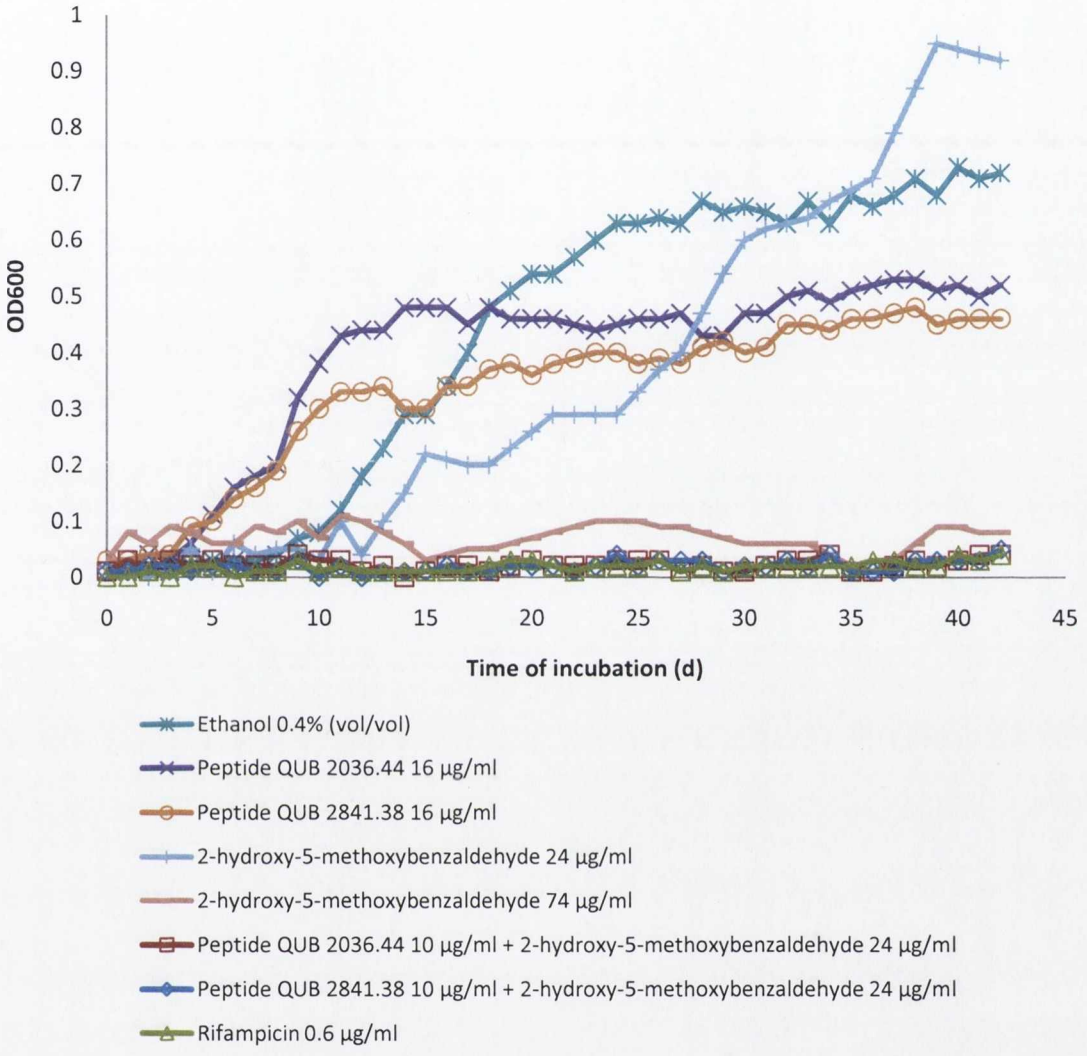

Figure 4.1.b Growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with the active compound 2-hydroxy-5-methoxybenzaldehyde at its MIC (74 µg/ml), one-third of its MIC (25 µg/ml), one-third of its MIC combined with non active peptide QUB 2036.44 (10 µg/ml) and QUB 2841.38 (10 µg/ml) respectively, QUB 2036.44 (16 µg/ml) and QUB 2841.38 (16 µg/ml) on their own, ethanol (0.4% vol/vol) (negative control) and rifampicin (0.6 µg/ml) (positive control). 2-hydroxy-5-methoxybenzaldehyde shows slightly higher initial OD values as it produced coloured solutions.

4.3.2 Combinations of amphibian peptides and naturally occurring compounds against *Map* using microbroth susceptibility testing method.

As shown in figures 4.2.a to 4.2.f, the microbroth dilution susceptibility testing results were expressed in terms of % reduction of alamarBlue reagent. The % reduction of alamarBlue reagent was used as an indicator of metabolic activity. For instance, if a low % reduction of alamarBlue reagent was observed, which indicated a low metabolic activity in the cytosol, thus suggesting that the antibacterial activity of the compound was strong. This method has been widely used in studies involving the growth of mammalian cells, it was also applied in studies of other *Mycobacterial* culture due to its rapid reaction, ability to scale down test materials, and performance in a high-throughput manner (Collins and Franzblau, 1997, Shingalapur et al., 2009, Silva et al., 2009).

As shown in figure 4.2.a, when alamarBlue was added to growth medium without any cells (negative), there was a small reduction (4%) in colour, this small reduction might due to colour change during the incubation period, but not the changes in metabolic activity. In the presence of positive control antibiotic rifampicin, the % reduction of alamarBlue was between 4-6%, comparing to the negative control, the difference is 0 to 2%, which indicated there was no or only very small metabolic activity, in the other word, the culture was effectively inhibited by the positive control antibiotic rifampicin. As shown in figure 4.2.b, in the presence of non-inhibitory peptides QUB 284138 and QUB 203644 and test wells with 0.4% vol/vol ethanol only, the % reduction was over 62%, which indicated a high metabolic activity of *Map* culture; as demonstrated in macrobroth dilution assay previously, the two amphibian peptides and 0.4% vol/vol ethanol did not inhibit the growth of *Map* culture.

As shown in figures 4.2.c to 4.2.f, when combining amphibian peptides QUB 284138 and QUB 203644 at 5, 10 and 20 μg/ml and the six anti-*Map* naturally occurring compounds at one-third of their MICs, there was no significant inhibition of growth of *Map*, comparing to the positive control rifampicin and supplementary positive control, anti-*Map* compounds at their MICs. These results were not in agreement with the previous macrobroth dilution susceptibility testing.

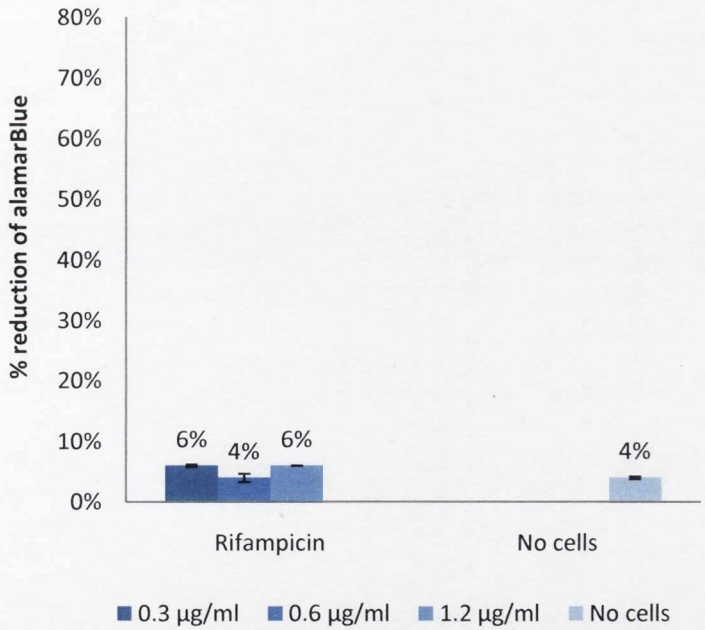

Figure 4.2.a Percentage reduction of alamarBlue cause by the growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with antibiotic rifampicin at 0.3, 0.6 and 1.2 µg/ml) (positive control) and % reduction of alamarBlue in Middlebrook 7H9 broth without addition of bacterial cells. Vertical bars indicate standard error of the mean (SEM).

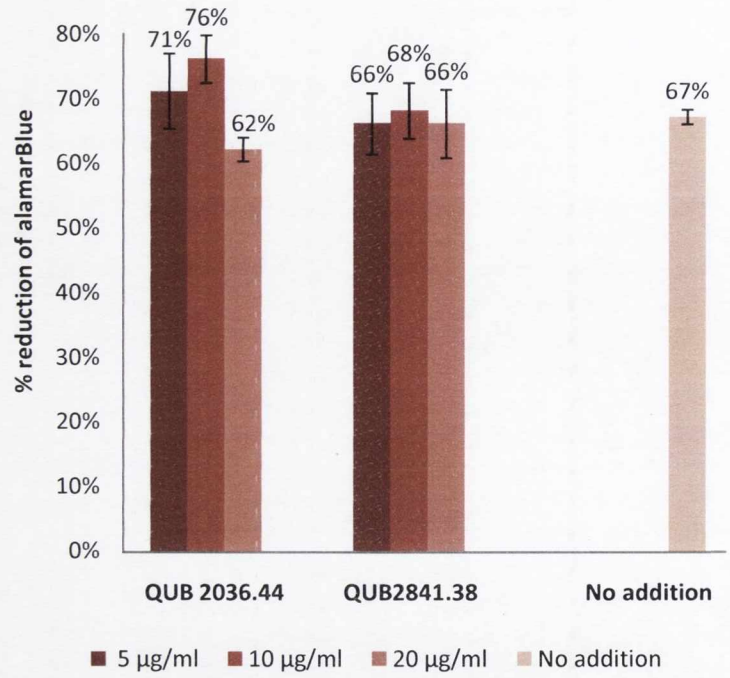

Figure 4.2.b Percentage reduction of alamarBlue cause by the growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with non-active amphibian peptides QUB 2036.44 and QUB 2841.38 on their own at concentrations 5, 10 and 20 µg/ml and without addition of any peptides. Vertical bars indicate standard error of the mean (SEM).

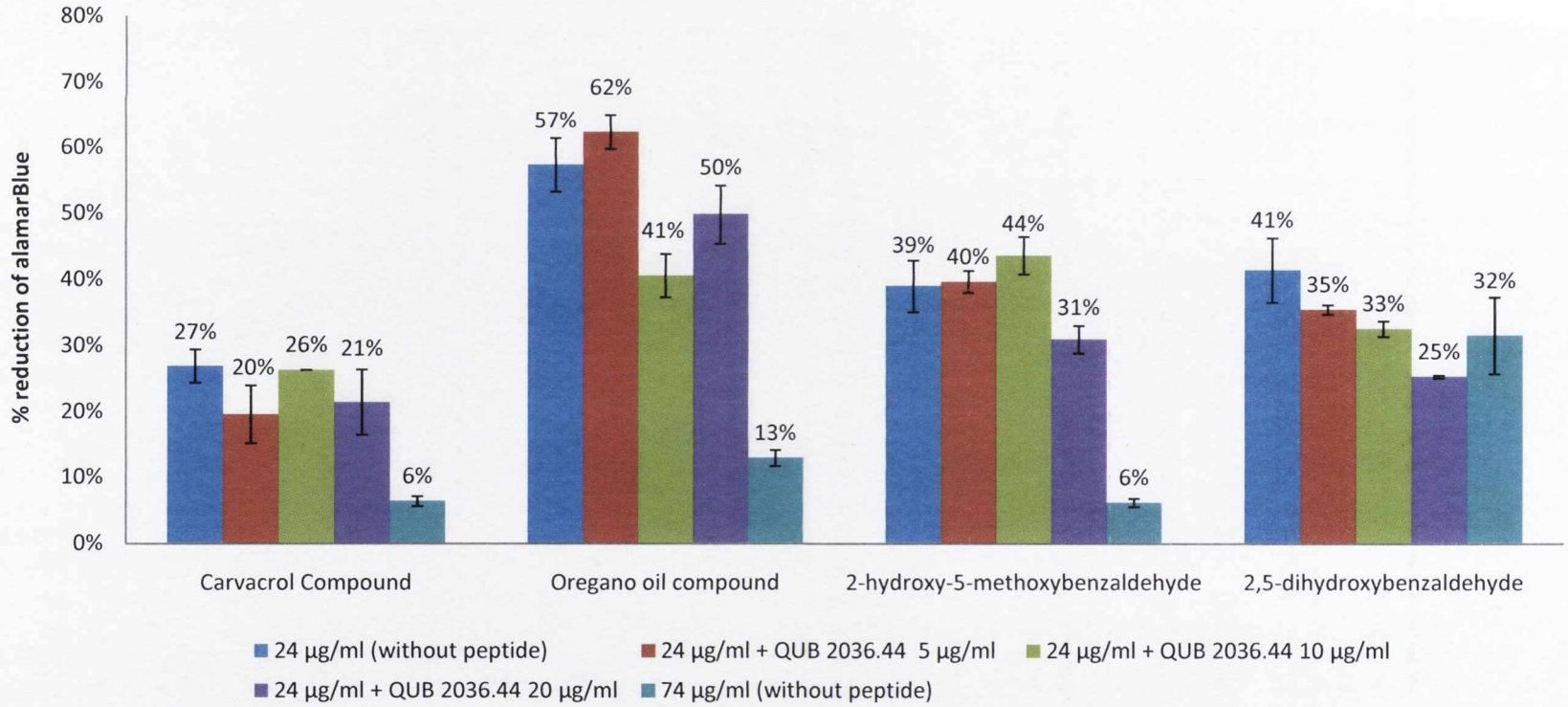

Figure 4.2.c Percentage reduction of alamarBlue cause by the growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with anti-*Map* naturally occurring compounds (carvacrol, oregano oil, 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde) on their own at one-third of their MIC: 24 µg/ml (supplementary negative control), combination of anti-*Map* naturally occurring compounds at one-third of their MIC: 24 µg/ml and non-active amphibian peptides QUB 2036.44 at concentrations 5, 10 and 20 µg/ml respectively and anti-*Map* naturally occurring compounds on their own at their MIC: 74 µg/ml without addition of any peptides (supplementary positive control). Vertical bars indicate standard error of the mean (SEM).

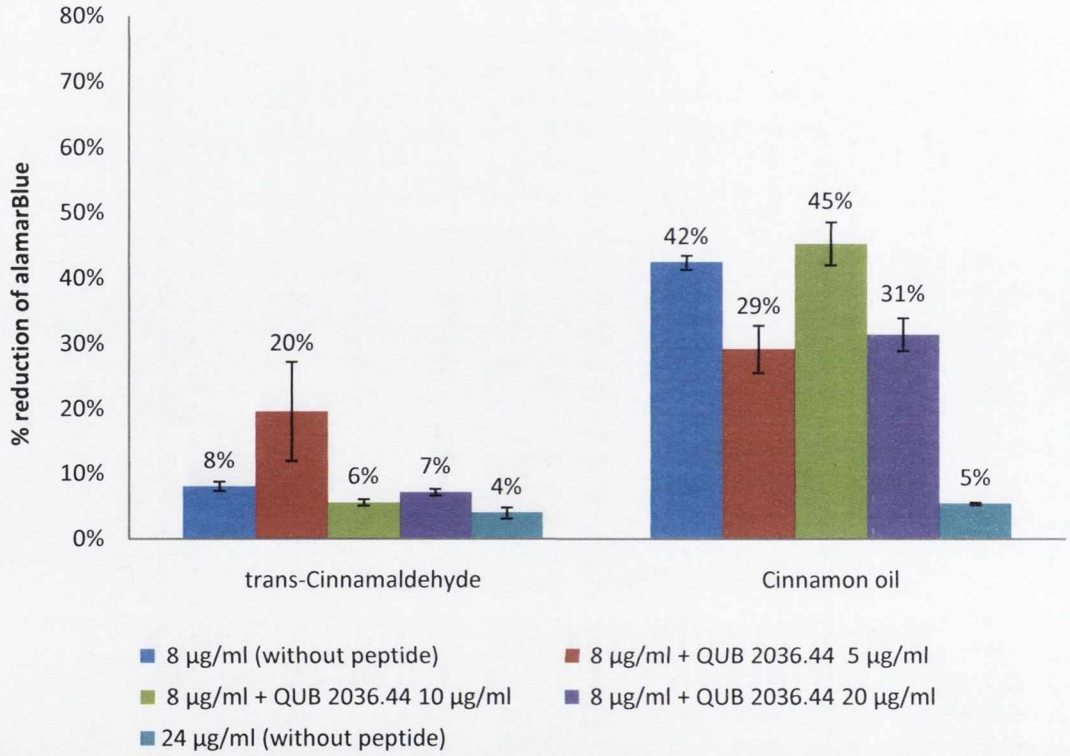

Figure 4.2.d Percentage reduction of alamarBlue cause by the growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with anti-*Map* naturally occurring compounds (*trans*-cinnamaldehyde and cinnamon oil) on their own at one-third of their MIC: 8 µg/ml (supplementary negative control), combination of anti-*Map* naturally occurring compounds at one-third of their MIC: 8 µg/ml and non-active amphibian peptides QUB 2036.44 at concentrations 5, 10 and 20 µg/ml respectively and anti-*Map* naturally occurring compounds on their own at their MIC: 24 µg/ml without addition of any peptides (supplementary positive control). Vertical bars indicate standard error of the mean (SEM).

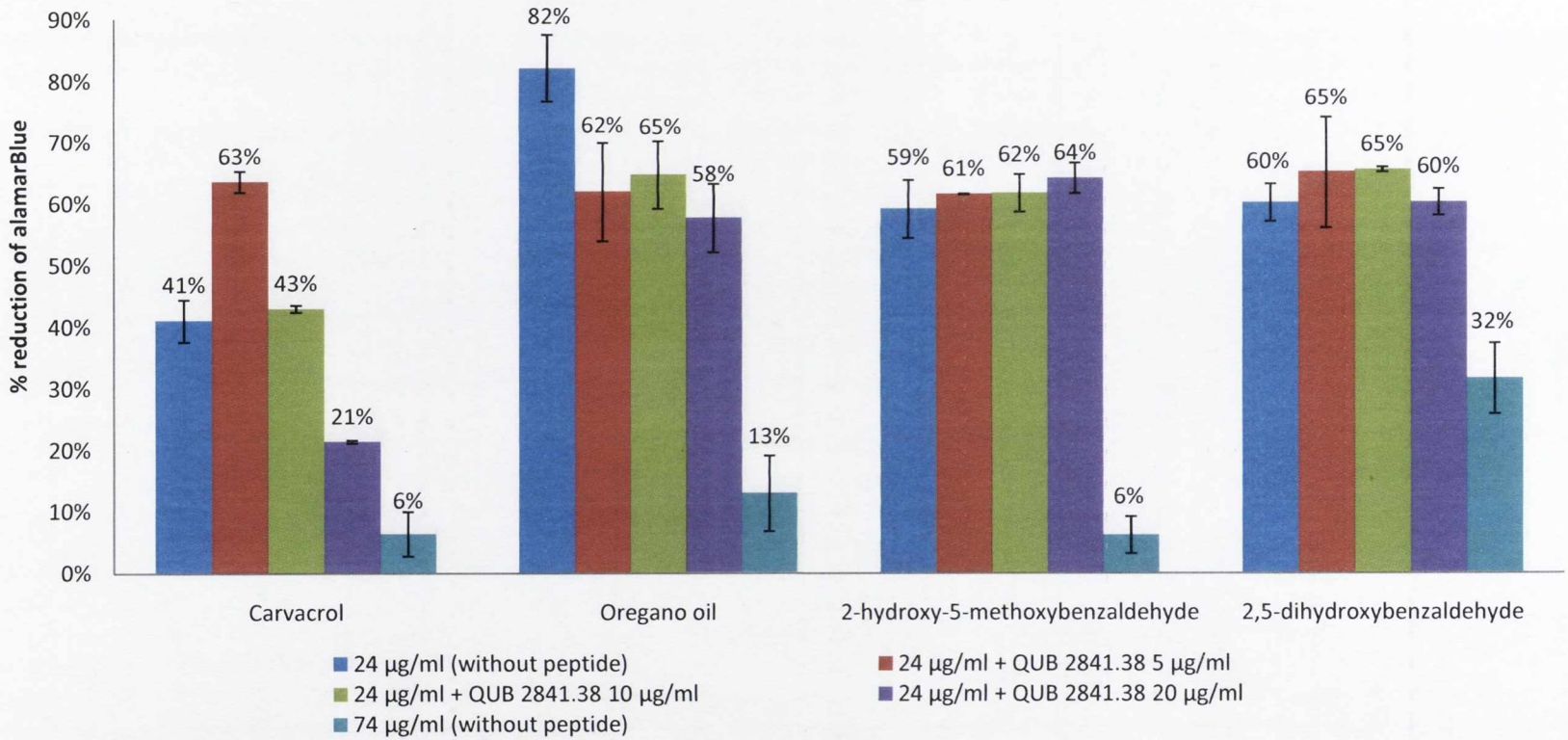

Figure 4.2.e Percentage reduction of alamarBlue cause by the growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with anti-*Map* naturally occurring compounds (carvacrol, oregano oil, 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde) on their own at one-third of their MIC: 24 µg/ml (supplementary negative control), combination of anti-*Map* naturally occurring compounds at one-third of their MIC: 24 µg/ml and non-active amphibian peptides QUB 2841.38 at concentrations 5, 10 and 20 µg/ml respectively and anti-*Map* naturally occurring compounds on their own at their MIC: 74 µg/ml without addition of any peptides (supplementary positive control). Vertical bars indicate standard error of the mean (SEM).

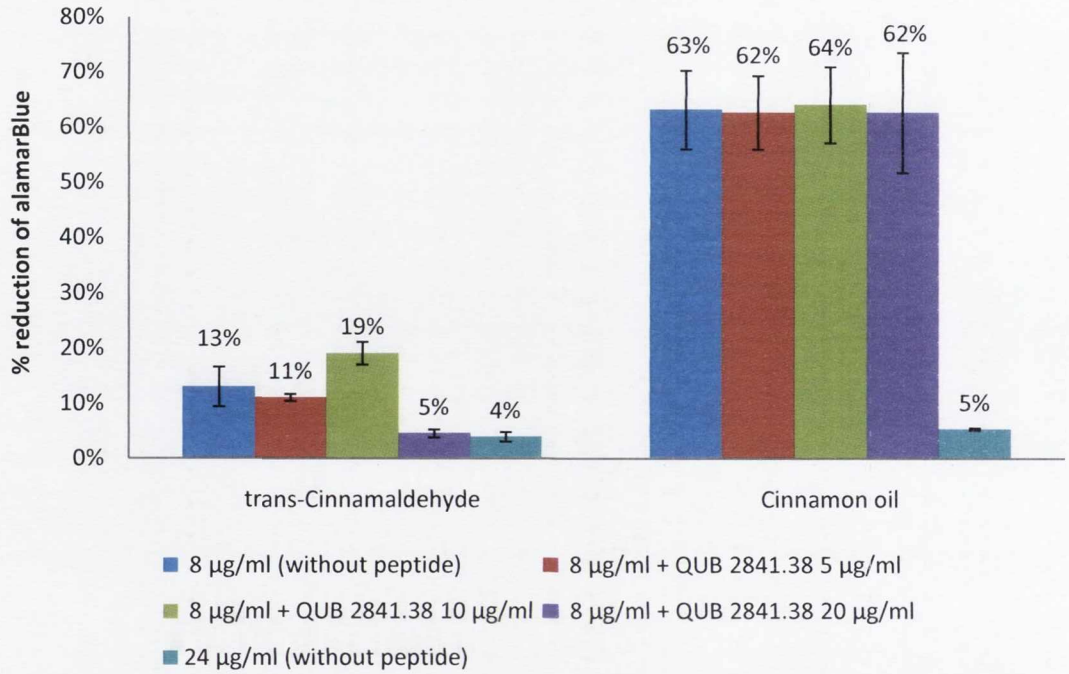

Figure 4.2.f Percentage reduction of alamarBlue cause by the growth of *M. avium* subsp. *paratuberculosis* NCTC 8578 in Middlebrook 7H9 broth supplemented with anti-*Map* naturally occurring compounds (*trans*-cinnamaldehyde and cinnamon oil) on their own at one-third of their MIC: 8 µg/ml (supplementary negative control), combination of anti-*Map* naturally occurring compounds at one-third of their MIC: 8 µg/ml and non-active amphibian peptides QUB 2841.38 at concentrations 5, 10 and 20 µg/ml respectively and anti-*Map* naturally occurring compounds on their own at their MIC: 24 µg/ml without addition of any peptides (supplementary positive control). Vertical bars indicate standard error of the mean (SEM).

4.3.3 Combinations of herbal extract Aw and naturally occurring compounds against *M. smegmatis* MC^2155 using macrobroth susceptibility testing method.

As shown in figure 4.3, when combining the naturally occurring compound, oregano oil at one-fourth of its MICs (74 μg/ml) and herbal extract Aw at one-third of its MIC (100 μg/ml), the growth of *M. smegmatis* MC^2155 was inhibited completely during incubation at 37°C for up to 10 days. This could have resulted from a partial synergistic effect or additive effect when combining the test agents together (Table 4.2).

Table 4.2 Calculation of FIC and FICI of antimycobacterial compounds and herbal extracts Aw against *M. smegmatis* MC^2155, method adopted from checkerboard assay.

Agent	MIC[a] (μg/ml)		FIC[b]	FICI[c]	Outcome
	Alone	Combination			
Aw	300	100	0.33	0.58	Partial synergistic
Oregano oil	296	74	0.25		

[a]MIC required to inhibit visible growth of the strain in test tube.

[b]FIC (fractional inhibitory concentration) of 1st compound = (MIC of 1st compound in combination) ÷ (MIC of 1st compound alone), and FIC of 2nd compound = (MIC of 2nd compound in combination) ÷ (MIC of 2nd compound alone), as described in Balke et al. (2006).

[c]FICI (fractional inhibitory concentration index) = (FIC of 1st compound) + (FIC of 2nd compound), it was defined as synergy ≤ 0.5; partial synergy FIC >0.5 but <1; additive FIC=1.0; indifferent effect when values were >1 and <4 and antagonistic when values were ≥ 4.0 as described in Kim et al. (2009) and Miranda-Novales et al. (2006).

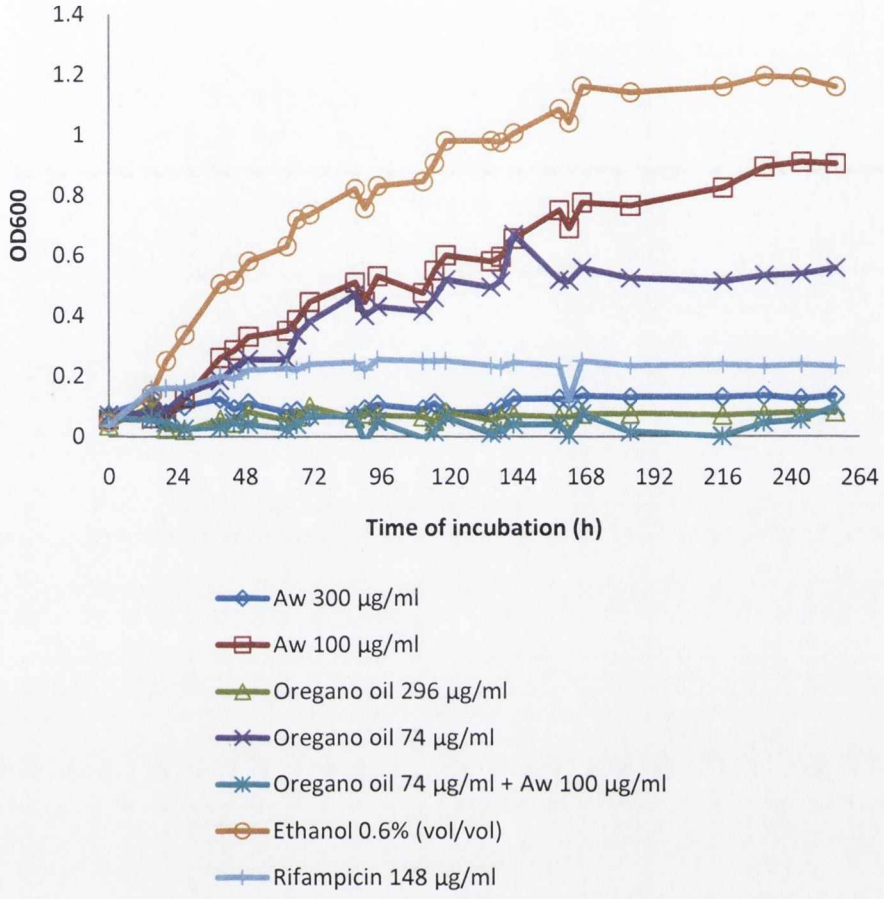

Figure 4.3 Growth of *M. smegmatis* MC^2155 in Middlebrook 7H9 broth supplemented with the active compound Aw at its MIC (300 µg/ml) and one-third of its MIC (100 µg/ml), oregano oil at its MIC (296 µg/ml) and one-fourth of its MIC (74 µg/ml), combination of oregano oil at one-fourth of its MIC (74 µg/ml) and Aw at one-third of its MICs (100 µg/ml), ethanol 0.6% (vol/vol) (negative control) and rifampicin (148 µg/ml) (positive control).

4.4 Discussion

4.4.1 Combination of amphibian peptides and naturally occurring compounds against *Map* using macrobroth susceptibility testing method

As shown in figures 4.1.a and 4.1.b, the growth of *Map* was inhibited when supplementing 5 μg/ml of non active amphibian peptides QUB 284138 and QUB 203644 with anti-*Map* compound 2,5-dihydroxybenzaldehyde at one-third of its MIC and 10 μg/ml of these two peptides with anti-*Map* compound 2-hydroxy-5-methoxybenzaldehyde at one-third of its MIC. This might have resulted from the synergistic effect of combining amphibian peptides and naturally occurring compounds together.

Amphibian peptides gain a high interest in the research of drug discovery due to their prominent antimicrobial effects and possible anti-cancer effects. Those peptides were secreted by the glands in the skin of the amphibians as part of their natural defence mechanism. The naturally occurring peptides used in our study originated found from frog skin; after being identified and characterised, the peptides were synthesized. The mode of action of cell death caused by amphibian peptides involves non-specific interaction with the membrane phospho/lipids. Both antimicrobial and cytolytic activities are relevant to the complex interaction between the cationicity, hydrophobicity, α-helicity and amphipathicity (Yeaman and Yount, 2003). The two main modes of actions of antimicrobial peptides were proposed as pore formation across the lipid bilayer (Ambroggio et al., 2005, Matsuzaki, 1998, Chen et al., 2003) and membrane disruption by tightly binding to the membrane interface (Ambroggio et al., 2005, Papo and Shai, 2003, Mani et al., 2004). It is believed that the N-terminal domain is important for determining antimicrobial activity due to its cationicity, which could target the cell surface through electrostatic interaction (Johnsen et al., 2005).

4.4.2 Combinations of amphibian peptides and naturally occurring compounds against *Map* using microbroth susceptibility testing method.

As shown in figures 4.2.c to 4.2.f, no significant inhibition of growth of *Map* was observed when combining amphibian peptides QUB 284138 and QUB 203644 at 5, 10 and 20 μg/ml and the six anti-*Map* naturally occurring compounds at one-third of their MICs, compared to the positive control rifampicin and supplementary positive control, anti-*Map* compounds at their MICs. These results were not in agreement with the previous macrobroth dilution susceptibility testing. The possible explanation for such variation might be due to a random mutation that occurred during experiment so the *Map* culture became more resistant to the combination of test compounds. Although the majority of mutations or drug resistance developed clinically during antibiotic treatment, spontaneous mutations also occur at a relatively low frequency (Chiang et al., 2010, David, 1970).

4.4.3 Combinations of herbal extract Aw and naturally occurring compounds against *M. smegmatis* MC^2155 using macrobroth susceptibility testing method.

Among the common methods used for determining synergistic effects, a checkerboard assay is the simplest and most popular (Odds, 2003). We adopted the formula to calculate fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) in the synergistic study of combination of naturally occurring compounds and herbal extract Aw only, but not in peptide synergistic study as the two amphibian peptides tested had not shown antimicrobial activity against *Map* at the highest test concentration (16 μg/ml), thus no MIC was determined.

As shown in figures 4.3, our results showed that combination of naturally occurring compound oregano oil and herbal extract Aw at sub-MIC concentrations had only a partial synergistic effect against *M. smegmatis* MC^2155, which is in good agreement of Nazer et al. (2005). The possible reason of the synergistic effect could be oregano oil had changed the integrity of the cell membrane of *M. smegmatis* MC^2155 due to its hydrophobicity, therefore resulting in cells being more susceptible to the herbal extract

Aw. The herbal extract Aw may also have a different antibacterial mode of action to *M. smegmatis* MC^2155. Further studies can be carried out to identify the active component(s) of the herbal extract Aw, which may help in understanding the finding of this experiment.

Nazer et al. (2005) demonstrated that combinations of essential oil components with organic acids, e.g. thymol and citric acid showed a better amplification of inhibition against *Salmonella typhimurium* in growth medium, however, when taking into account the cumulative effect of each compound in combinations; the researchers did not consider that was a real synergistic effect. The reason for that might be because the antimicrobial compounds exert their effects via similar modes of action rather than distinctive ones. Nevertheless, even an additive effect against microorganisms might still be beneficial when applying in food preservation technology because when compounds are used in combination at lower concentrations, no one single component is dominate, thus a better sensory quality of the food products might be maintained.

Recently it has been suggested that the mechanism of synergism may be due to one compound altering the membrane integrity, thus increasing the uptake of other antimicrobial compounds (Tokarskyy and Marshall, 2008, Kim et al., 2009). Another mechanism may be that one compound blocks one of the enzymes for protein synthesis, while the second compound may inhibit sequential steps in protein synthesis (Ulvatnea et al., 2001). Combining antimicrobials that exert synergistic effects could maximize the use of such agents and help to reduce the cost in some cases. Numerous on-going research projects have been carried out on the study of possible synergistic effects such as the study by Zhou et al. (2007) is among those successful examples. In this study, essential oil components (thymol and carvacrol) were combined with other food preservatives (e.g. chelator EDTA) and organic acids (e.g. acetic acid and citric acid), synergistic effects were observed against *Salmonella typhimurium* in growth medium. The MICs of thymol and carvacrol were decreased by 4-fold in the addition of acetic acid. The researchers concluded that the results might due to EDTA increasing the sensitivity of bacteria to the essential oil components and organic acid changing the

equilibrium, thus allowing essential oil components to exist in a form that can enter bacterial cells and exert antimicrobial activity.

To conclude, the possible synergistic effect of a combination of anti-*Map* naturally occurring compounds and amphibian peptides against *Map*, and a combination of naturally occurring compounds and herbal extract of *Coptis chinensis Franch* (Aw) against *M. smegmatis* MC^2155 was studied. The amphibian peptides showed no antimicrobial effect against *Map* even at the highest concentration tested but could lower the MICs of naturally occurring compounds 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde in the macrobroth susceptibility testing. This effect, however, was not observed in the microbroth susceptibility testing. The possible reason for such a variation might be due to random mutation which occurred during the experiment, making the *Map* culture more resistant to the combination of test compounds. Therefore, it is not possible to confirm the existence of a synergistic effect. Nevertheless, when combining naturally occurring compounds, oregano oil and herbal extract Aw at sub-MIC concentrations, a partial synergistic effect was observed. The impact of synergistic effects on applications can be, to reduce the amount used, the cost, and possible cytotoxicity of antimicrobial natural compounds. The additive effect will also be beneficial as it can reduce the amount of natural compounds used and maintain the sensory acceptance of food products when applied in food preservation technology.

5 Determination of possible modes of antimicrobial action of naturally occurring compounds against *Mycobacterium avium* subspecies *paratuberculosis* and *Escherichia coli*

5.1 Introduction

In the modern food industry, food safety and quality are becoming the major concerns as the food-health interface can lead to very different consequences, both negative and positive. Consumers demand food that not only meets nutritional standards but is also of high quality, safe and hazard-free, thus mild preservation technologies have become more popular as minimally processed food can retain their natural values as well as to extend the shelf life of products, e.g. flavour, colour texture and nutrition (Ohlsson and Bengtsson, 2002). To achieve this, several approaches are usually combined and one of these is the addition of natural preservatives to prevent food spoilages caused by microorganisms such as bacteria and fungi (Ultee et al., 1999). Many naturally-occurring compounds, such as plant extracts and their secondary metabolites, essential oils and their isolated constituents, and phenolic compounds have been evaluated extensively for antimicrobial activity and are chemically characterized. The application of naturally occurring compounds is drawing much attention in food industry research due to their prominent antimicrobial activities against a broad range of microorganisms relevant to food safety and quality, including both Gram positive and Gram negative bacteria. For example *Bacillus cereus* (Ultee et al., 1999, Friedman et al., 2006b), *Listeria monocytogenes* (Gill and Holley, 2004, Je and Kim, 2006), *Pseudomonas aeruginosa* (Je and Kim, 2006, Lambert et al., 2001) *Staphylococcus aureus* (Je and Kim, 2006, Lambert et al., 2001), *Escherichia coli* (Fitzgerald et al., 2004, Helander et al., 1998, Je and Kim, 2006) and *Salmonella typhimurium* (Je and Kim, 2006). Some natural compounds, e.g. cinnamon oil and oregano oil have also shown effects on antibiotic resistant pathogens include *B. cereus*, *E. coli* and *S. aureus* (Friedman et al., 2004a). Naturally occurring compounds are ideal for study as most of them are 'generally recognised as safe' (Kabara, 1991) and they had been traditionally used as herbs and spices for enhancing the flavour of food as well as folk medicines in our history.

Investigation into the mode of action of naturally occurring compounds is of special interest and importance to food safety research and development of foods of high standard of safety and quality. An understanding of the mode of action of antimicrobials can help to design and develop new drug candidates with high specific activity against certain microorganisms (Epand and Epand, 2009b). It may also help to modify existing drugs to maximise their pharmaceutical potencies. Moreover, combining several antimicrobial compounds with different modes of action may generate a synergistic effect, which can help to reduce the amount of compounds used and thus the cost required in food preservation by means of inhibiting the growth of microorganisms. Furthermore, as many natural compounds, especially essential oils may have their own distinctive smells, tastes and colours, when applying them as food preservatives, it is important to keep them at as low concentrations as possible to minimise the alteration of natural quality of food products and meet consumers' acceptance on organoleptic quality (Lambert et al., 2001).

Antimicrobial activities of naturally occurring compounds, especially essential oils and their components are generally known to target the cell membrane of microorganisms owing to their hydrophobic nature, which enables them to partition into the hydrophobic part of the phospholipid bilayer and accumulate at cell membrane. As a consequence, the cell membrane loses its integrity and membrane associated events could occur (Sikkema et al., 1995). The membrane plays an important role in keeping cell's integrity and viability due to its vital functions. Firstly its barrier function, secondly energy transduction which allows the membrane to form ion gradients channels that can be used for various metabolic processes and finally its service as a matrix for membrane-embedded proteins which are important for metabolic activities, e.g. ATP-synthase (Sikkema et al., 1995).

However, the precise molecular interactions between the naturally occurring compounds and the bacterial cells still remains vaguely defined (Lambert et al., 2001). It is believed that naturally occurring antimicrobial compounds might exert several modes of action to

achieve metabolic inhibition, growth inhibition of microbes and finally cell death (Burt, 2004). Current methods for studying antibacterial mechanisms include measurement of changes of cell homeostasis, e.g. use of fluorescent probes to measure the change of intracellular pH and relative change of membrane potential (Ultee et al., 1999). Measurement of oxygen consumption is another parameter, i.e. respiration of cells using oxygen probes (Fitzgerald et al., 2004) and proteomic studies to investigate any protein expression or repression under stressful but non-lethal antimicrobial treatments. Observations of changes in cell morphology after antimicrobial treatment using transmission and scanning electron microscopy; measurement of cell membrane stability, e.g. whole cell autolysis, which could indicate if the antimicrobials cause disintegration of cells; uptake of fluorescent probe NPN to cell membranes (Helander et al., 1998) have all been employed. The measurement of intracellular constituents in the extracellular environment, e.g. phosphate and potassium ions, are good indicators of membrane leakage and can reflect the severity of membrane damage. Such cellular constituents are important for cellular functions thus leakage of these constituents might cause further disturbances in cellular metabolism and viability.

Many recent studies have focused on a series of cellular responses, especially leakage studies as it may help to assess the degree of membrane damage, membrane permeability and membrane stability. These can be achieved through the measurements of different intracellular constituents, e.g. soluble proteins, nucleic acid, phosphate ion and potassium ion, intracellular pH, proton motive force and effect on ATP production (Je and Kim, 2006, Lambert et al., 2001, Helander et al., 1998, Ultee et al., 1999, Gill and Holley, 2004).

Experiments performed in this study include whole cell autolysis, measurement of extracellular phosphate, protein and nucleic acid, measurement of intracellular and extracellular ATP and absorbance scans of culture supernatant after antimicrobial treatment for screening of leakage of other possible constituents.

The previously outlined studies and many other separate studies were carried out on common food pathogens. In the present study, *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) was selected as it is not only a food pathogen but also a disease pathogen both in animals and humans. The most common foodborne pathogen *Escherichia coli* was also included and non-pathogenic *E. coli* K12 was used as a surrogate for the pathogenic O157:H7 strain. *E. coli* could cause fatality in severe cases. To our knowledge, this is the first study to determine the cellular responses and possible modes of action of naturally occurring compounds against *Map* and the methods, whole cell autolysis and determination of extracellular concentration of phosphate ions adopted in our study are novel in determining the mode of action of these naturally occurring compounds against *E. coli*.

5.2 Materials and methods

5.2.1 Test compounds

The following naturally occurring compounds were originally obtained from Sigma (St. Louis, MO): 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, caffeic acid, capsaicin (natural), carvacrol, chlorogenic acid hemihydrates, *trans*-cinnamaldehyde, citral (*cis* + *trans*), gallic acid, geraniol, methyl cinnamate and vanillic acid. The purity levels of these compounds ranged from 95 to 99.9% according to the manufacturer. Cinnamon oil (Cassia) was originally obtained from Yerba Buena Co. (Berkeley, Calif.). Oregano oil (Origanum) was originally obtained from Lhasa Karnak Herb Co. (Berkeley, Calif.). Their purity levels were not specified. Apple E (concentrated apple polyphenols) was originally obtained from Apple Poly LLC (Morrill, NE); the purity level was approximately 82%. Green tea polyphenols were originally obtained from LKT Laboratories, Inc. (St. Paul, MN); the purity level was not specified. Garlic powder and onion powder (cooking ingredients) were originally obtained from McCormick & Company Inc. (Sparks, MD). Their purity levels were not specified. The above compounds were gifts from Dr Mendel Friedma, Western Regional Research Center, U.S. Department of Agriculture, Albany, California, USA. Gentamycin and rifampicin (Sigma) were used as positive control antibiotics as it is known to be active against *E. coli* and *Map* respectively.

5.2.2 Preparation of test compounds

Stock solutions, 50 mg/ml for solid compounds and 50 µl/ml for oil compounds were prepared by suspension in absolute ethanol. Rifampicin was prepared as a 10 mg/ml stock suspension in absolute ethanol. The stock solutions were stored in aluminium foil-wrapped bottles at 4°C. As a high percentage of ethanol could be bactericidal, the maximum concentration of ethanol that a strain could tolerate (showing no observable inhibition of growth) was determined in the preliminary study of bactericidal assay in previous chapter. The final concentration of ethanol present in all studies for *Map* was standardized at 0.4% (vol/vol) and for *E. coli* was standardized at 1% (vol/vol) or otherwise specified.

5.2.3 Bacterial strains and growth conditions

Map NCTC 8578 (a bovine isolate) and *E. coli* K12 NCTC 10538 were obtained from the National Collection of Type Cultures, Colindale, London. The strains were maintained and cultivated as described in Chapter 2 and Chapter 3 in this thesis. When a larger volume of *E. coli* culture was required, 1 ml of the above culture was used to inoculate 100 ml of nutrient broth and incubated in the same conditions.

5.2.4 General treatment to harvest bacterial cultures

Fifty ml of early stationary phase *Map* culture (OD_{600} = 0.7 – 1.0, cfu/ml = ~ 5 x 10^7) was spun at 4,400 rpm for 30 min to pellet the cells. Supernatant (45 ml) was decanted carefully to avoid disturbing the cell pellet. Ten glass beads (3 mm) were added to the centrifuge tube and vortexed at high speed for 2 min to de-clump the culture; it was then rested for 2 min and repeated 3 times. Cell pellets were washed three times in sterile reverse osmosis water at room temperature. Cell pellets were de-clumped again at the final washing step if necessary (as *Map* tended to clump in solution without Tween 80).

Fifty ml of early stationary phase *E. coli* culture (OD_{600} = ~1.6, cfu/ml = ~ 2.3 x 10^8) was spun at 4,400 rpm for 10 min to pellet the cells. Supernatant was decanted carefully to avoid disturbing the cell pellet. The pellet was washed three times in sterile reverse osmosis water.

5.2.5 Whole cell autolysis assay

Whole cell autolysis assay was carried out as described by Gustafson et al. (1998), with some modifications. The washed *Map* cell pellet was resuspended in sterile buffer (50 mM Tris-HCl) supplemented with 0.5% Tween 80 (Sigma, UK) and the OD_{600} was adjusted to 0.25. The resultant cultures were dispensed into sterile test tube (3 ml each) containing the six anti-*Map* compounds and antibiotic rifampicin at their MICs (cinnamon oil: 24 µg/ml and its constituent cinnamaldehyde: 24 µg/ml, oregano oil: 74 µg/ml and its constituent carvacrol: 74 µg/ml, 2,5-dihydroxybenzaldehyde: 74 µg/ml, 2-hydroxy-5-methoxynenzaldehyde: 74 µg/ml and rifampicin: 0.625 µg/ml. The final

concentration of ethanol was 0.4% (vol/vol). The cultures were then incubated at 37 °C, OD_{600} reading of the test tubes was measured every 2 h over the first 3 days then daily for up to 17 days. All test tubes were prepared in duplicate.

The washed *E. coli* cell pellet was resuspended in sterile 50 mM Tris-HCl buffer (pH 7.0) and the OD_{600} was adjusted to 0.8. The adjusted cultures were dispensed into sterile test tubes (3 ml each) containing test compounds. All the naturally occurring compounds were tested at 500 µg/ml. The final concentration of ethanol was 1% (vol/vol). Non-bactericidal compound vanillic acid and ethanol 1% (vol/vol) were included as negative controls and gentamycin was used as positive control. The cultures were incubated at 37 °C, OD_{600} reading of the test tubes was measured hourly from time 0 h to 7 h and a final reading was taken at 24 h. As some naturally occurring compounds formed emulsions or changed colour during the incubation period, which contributed to the variations of the OD_{600} readings, corresponding blanks prepared from buffer and test compounds only but without bacterial cultures were incorporated for correction of the colour changes. All test tubes were prepared in duplicate and the experiment was repeated three times.

5.2.6 Determination of extracellular phosphate concentration

The Phosphate assay was carried out as described by Lambert et al., (2001), with the following modifications. The washed *Map* cell pellets were resuspended in sterile HPLC water (Sigma, UK) in the original volume (HPLC water was used in this experiment but not buffer as the salts in buffer might interfere the colour development of phosphate assay reagent); aliquots were made into sterile small bottles and added with test compounds. Four of the anti-*Map* compounds (cinnamaldehyde, carvacrol, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxynenzaldehyde) and non active compound vanillic acid were tested at 8, 24, 74, 222 and 1,000 µg/ml. Ethanol (0.4%, vol/vol) was used as negative control. The bottles were incubated at 37 °C for 1 d. At time 0, 1, 2, 4, 8 and 24 h, aliquots were taken and pipetted into eppendorf tubes and centrifuged at 14,000 rpm for 15 min, supernatants were carefully taken and pipetted into fresh eppendorf tubes. All samples were stored at -20°C prior to testing.

Before the test, samples were brought to ambient temperature, 50 µl of each samples were piepetted into clear polystyrene flat bottom 96-well plate (Sarstedt Ltd, Leicester, UK), 100 µl of Biomol Green™ phosphate assay reagent (Enzo Life Sciences UK Ltd., Exeter, UK; *formerly* Biomol International LP) was dispensed into each well using multichannel pipette. The plate was sealed with a plate sealer and incubated at room temperature for 20 min for colour development and the OD_{620} was measured using a microplate reader (Tecan UK Ltd, Reading, UK). Phosphate standard solution (Enzo Life Sciences UK Ltd., Exeter, UK; *formerly* Biomol International LP) was prepared to establish a calibration curve range from 0 - 40 µM for quantification of phosphate concentration.

For determination of extracellular phosphate concentration of *E. coli* culture, the experimental procedures were the same as above, except the compounds tested were bactericidal against *E.* coli at relatively low concentrations, which were 2,5-dihydroxybenzaldehyde, apple E polyphenols, gallic acid, green tea polyphenols, garlic powder, onion powder and oregano oil. Only one concentration was tested, which was their MICs, along with negative controls ethanol and vanillic acid. The samples were taken at different time intervals, which were 0, 15, 30, 60 and 120 min.

5.2.7 Determination of extracellular amount of nucleic acid

The possible leakage of nucleic acids to the extracellular environment was investigated using procedures described by Je and Kim (2006), with some modifications. The washed *Map* cell pellets were resuspended in sterile reverse osmosis water in the original volume; aliquots were made into sterile small bottles and added with test compounds. Four of the anti-*Map* compounds (cinnamaldehyde, carvacrol, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxynenzaldehyde) were tested at 24, 74 and 222 µg/ml in duplicate and the bottles were incubated at 37 °C for 3 d and repeated two times. At time 0, 0.5, 1, 2, and 3 d, aliquots were pipetted to eppendorf tubes for centrifugation, samples were spun at 550 g for 15 min, and supernatants were carefully taken and pipetted into fresh eppendorf tubes. All samples were stored at -20°C prior to testing.

Before the test, samples were brought to ambient temperature. As most of the naturally occurring compounds absorb UV, samples of supernatant were subjected to nucleic acid precipitation to determine the quantity of extracellular nucleic acid. For each sample, 500 µl of supernatant was pipetted into an eppendorf tube, same volume, i.e. 500 µl of phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma, UK) was added to the supernatant. The mixture was vortexed for 1 min and spun at 10,000 rpm for 15 min. The aqueous layer (top layer) was transferred to a new ependorf tube, 60% volume, i.e. 300 µl of isopropanol was added to the aqueous layer and vortexed for 1 min. The mixture was stored at -20 °C overnight and spun at 14,000 rpm for 15 min. The supernatant was decanted carefully. The pellet (invisible) was washed using 70% ethanol and then recovered by spinning at 14,000 rpm for 15 min. The 70% ethanol was tapped out carefully. The pellet was allowed to air dry in the fume cupboard overnight. Finally 50 µl of 10 mM TE buffer was added to resuspend the precipitated nucleic acid. Concentration of DNA was obtained directly by placing the sample into UVette® (Eppendorf UK Ltd, Cambridge, UK) and measured in BioPhotometer (Eppendorf UK Ltd, Cambridge, UK). Calibration was not required for the quantification of DNA concentration.

5.2.8 Determination of extracellular amount of soluble protein

The possible leakage of soluble protein to extracellular environment was investigated using the method as described by Helander et al. (1998), with the following modifications. The washed *Map* cell pellets were resuspended in sterile reverse osmosis water in the original volume; aliquots were made into sterile small bottles and added with test compounds. Four of the anti-*Map* compounds (cinnamaldehyde, carvacrol, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde) were tested at 74 µg/ml in duplicate and the bottles were incubated at 37 °C for 2 d and repeated two times. At time 0, 0.5, 1, 1.5, and 2 d, aliquots of samples were taken and pipetted into eppendorf tubes and centrifuged at 550 g for 15 min, supernatants were removed carefully and transferred to fresh eppendorf tubes. All samples were stored at -20°C prior to testing.

Before the test, samples were brought to ambient temperature. For each 800 µl of test sample, 200 µl of Bio-Rad protein assay dye reagent was added and incubated at room temperature for 10 min. The absorbance at 595 nm (A_{595}) was measured using a BioPhotometer (Eppendorf UK Ltd, Cambridge, UK). BSA standard (1 - 25 µg/ml) was used to establish a calibration curve for quantification of protein concentration in the samples. As some natural compounds formed coloured complex with protein dye, which contributed higher readings at the A_{595}, corresponding blanks prepared with water and test compounds only (without bacterial cultures) were included in the experiment for correction of the colour changes. All test tubes were prepared in duplicate and the experiment was repeated two times.

5.2.9 Determination of intra- and extracellular ATP concentration

The intracellular and extracellular concentrations of ATP were investigated with the procedures described by Gill and Holley (2004), with the following modifications. Briefly, the washed *Map* pellet was resuspended in 45 ml of 7H9 broth base with 0.05% Tween 80 only but not OADC or mycobactine J and incubated at 37°C to deplete the intracellular ATP for 6 weeks (approximately one growth cycle of *Map*). The cell suspension was aliquot into small bottles, 3.6 ml each, and supplemented with 0.5% D-glucose (by adding 400 µl of 5% sterile D-glucose stock solution). Each bottle was added with 17.76 µl of natural compound stock in duplicate to achieve the MIC. (Cinnamaldehyde: 74 µg/ml, cinnamon oil: 74 µg/ml, carvacrol: 74 µg/ml, oregano oil: 74 µg/ml, 2,5-dihydroxybenzaldehyde: 74 µg/ml and 2-hydroxy-5-methoxybenzaldehyde: 74 µg/ml). Two of the bottles were added with 17.76 µl of 2,4,6-trihydroxybenzaldehyde (a non-active compound) stock to achieve final concentration of 74 µg/ml. Another two bottles were added with 17.76 µl of absolute ethanol (final concentration 0.4% vol/vol) to serve as negative controls, as all the compound stocks were prepared in absolute ethanol.

At time 0, 1, 2, 4, 8 and 24 h, samples were taken and subjected to the following preparation. A 500 µl-sample from each bottle was transferred to an eppendorf tube; the

cells were pelleted by spinning at 14,000 rpm for 15 min, followed by carefully transferring of 250 µl of the supernatant (avoid disturbing the pellet) to an eppendorf tube containing 250 µl of 200 mM Tris + 4 mM EDTA + 0.05% DTAB (for stabilization and determination of extracellular ATP concentration). The remaining supernatant was discarded carefully, the cell pellet was resuspended in 500 µl of extractant (100 mM Tris + 2 mM EDTA + 0.025% DTAB). The cell suspensions and supernatant mixtures were heated in a boiling bath for 20 min for cells inactivation and ATP extraction. After heat treatment, samples were placed in ice bath to facilitate quick cooling. Samples were then stored at -20°C before ATP assay.

Prior to the ATP assay, samples were thawed at ambient temperature. An aliquot of 50 µl of each sample was placed in a white 96-well plate (Greiner Bio-One Ltd, Gloucestershire, UK) in triplicate. ATP standard (Sigma, UK) 1, 2, 4, 8, 16, 32 and 64 nM prepared in extractant was used to establish a calibration curve. Fifty µl of the ATP assay mix reagent (Sigma, UK) 25-fold diluted (according to manufacturer) was dispensed to each sample and standard, and the bioluminescence was read immediately using a micro-plate reader (Mithras LB 940, Berthold Technologies UK Ltd, Herts, UK).

5.2.10 Absorbance scans of culture supernatant after antimicrobial treatment

The washed *Map* cell pellets were resuspended in sterile reverse osmosis water in the original volume; aliquots were prepared in small sterile bottles and added with test compounds. Four of the anti-*Map* compounds (cinnamaldehyde, carvacrol, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxynenzaldehyde) and non active compound vanillic acid were tested at 74 µg/ml, except cinnamaldehyde was tested at 24 µg/ml. Ethanol 0.4% (vol/vol) was used as a negative control, as all the natural compounds were suspended in absolute ethanol as a stock solution prior to addition to the *Map* cultures. The bottles were incubated at 37 °C for 4 d. At time 0, 1, 2, 4, 8, 24, 48, 72 and 96 h, sample aliquots were taken and pipetted into eppendorf tubes and for centrifuged at 14,000 rpm for 15 min. Supernatants were taken and pipetted carefully into fresh eppendorf tubes. All samples were stored at -20°C prior to testing.

Before the test, samples were brought to ambient temperature, 200 µl of each samples were pipetted into flat bottom 96-well UV plate (Costar, Corning Ltd, Sunderland, UK). The samples were scanned from 230 nm to 450 nm, the wavelength step size was 5 nm, using a microplate reader (Tecan UK Ltd, Reading, UK). As most of the natural compounds absorb UV light and may show more than one peak when scanned for a series of wavelength; and also they might not be stable when incubated at 37°C, a corresponding blank (composed of water and natural compound only, without *Map* culture) was prepared for each natural compound and the corresponding blank was scanned and compared to the supernatant sample.

5.2.11 Data analysis

All the results were recorded and processed using a Microsoft Office Excel spreadsheet to generate calibration curves and graphs for quantification of samples. Graphs were plotted using the mean of the replicates or triplicates of the experiments.

5.3 Results

5.3.1 Whole cell autolysis assay

Effects of the addition of six anti-*Map* naturally occurring compounds on *Map* cell lysis are shown in figure 5.1.a. Ethanol (0.4%, vol/vol) was used as negative control. The results indicated that a general decrease (20 %) in OD_{600} measurements occurred across all test samples including both active compounds and negative control ethanol. The highest decrease in OD_{600} measurement was observed in culture incubated with cinnamaldehyde, followed by negative control ethanol, the differences between samples were not appreciable.

Figure 5.1.b shows the effect of various naturally occurring compounds which exist in powder forms as compared to the negative control, 1% (vol/vol) ethanol on *E. coli* culture. The results showed that a general change of ~ 20 % (either increase or decrease) in OD measurements appeared across all test samples including active and non-active compounds, as well as the negative control ethanol. The increase of OD_{600} reading might be due to a change of colour of the naturally occurring compounds during incubation. The highest decrease of OD_{600} was observed in negative control ethanol, which might be caused by death and lysis of a portion of the cells in the test tubes, the differences between samples were not significant. The culture incubated with garlic powder showed some variations (a drop at time 2 h and then a rise at time 6 h) of OD_{600}, compared to the culture incubated with other powdered compounds, such variations might due to instrument error or a small defect on the test tube.

Figure 5.1.c shows the effect of the addition of various naturally occurring compounds, presented as oils compared with the negative control, 1% ethanol (vol/vol) in OD measurements of on *E. coli*. Similar to the powdered compounds, the results showed that a change of ~20% (either increase or decrease) in OD_{600} measurements were found in all test samples including active and non-active compounds and negative control ethanol; and the highest decrease of OD_{600} was observed in negative control ethanol. The increase of OD_{600} reading might be due to the formation of emulsion of the oil compounds during

incubation. The differences between samples might be due to different degree of emulsion formed in the test tubes.

The whole cell autolysis of *E. coli* was tested on all the naturally occurring compounds that show bactericidal effects on *E. coli* in our previous study including those with low and high MICs. Results of some naturally occurring compounds were not presented in the graphs as strong coloured solutions and emulsions were developed, leading to out of scale OD measurements. The corresponding blanks prepared without bacterial cultures did not develop to the same degree of turbidity. It might be because the colour change was partially attributed by metabolism of bacteria in cultures.

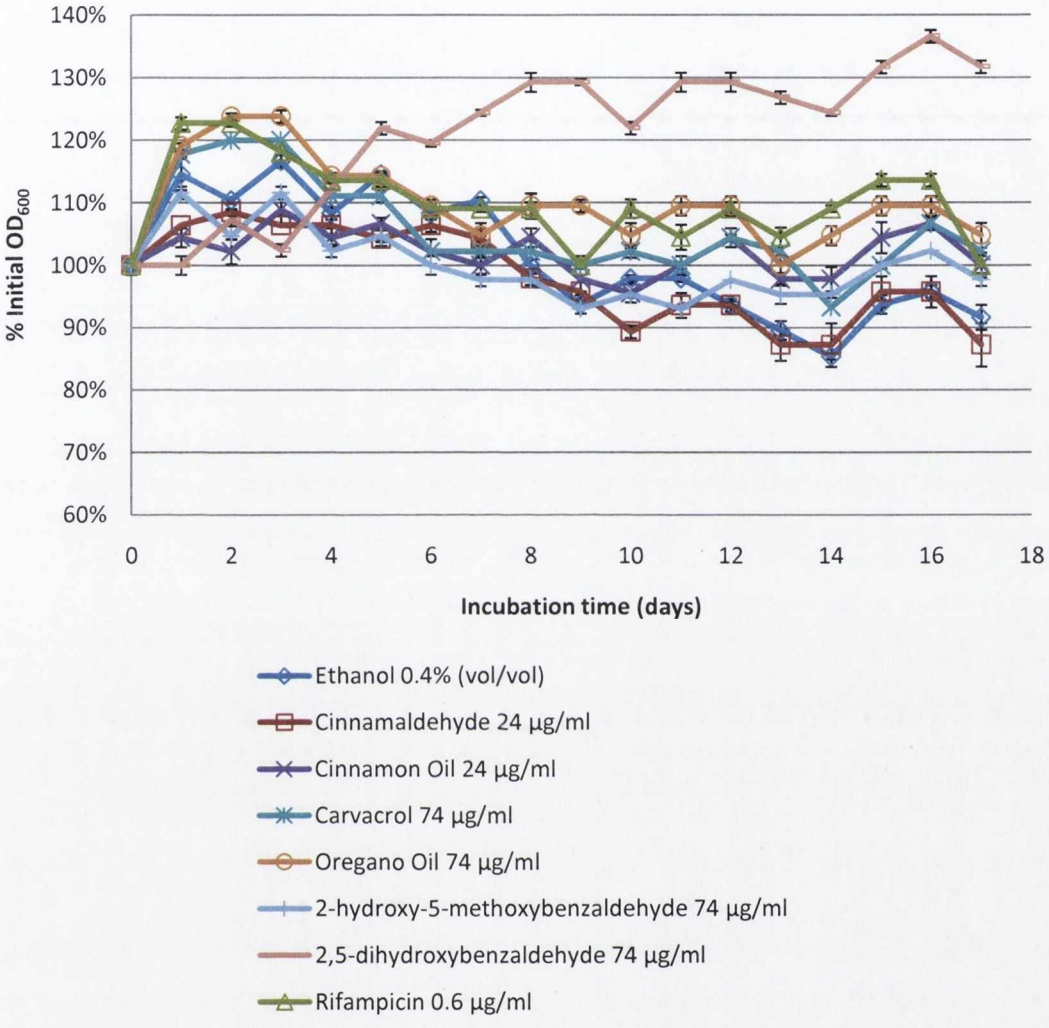

Figure 5.1.a Effect of naturally occurring compounds at their MICs and non-inhibitory concentration of ethanol (0.4% vol/vol, negative control) on *Map* culture. Test tubes containing 2,5-dihydroxybenzaldehyde showed an increase in %OD which may due to the coloured solution produced. Vertical bars indicate standard error of the mean (SEM).

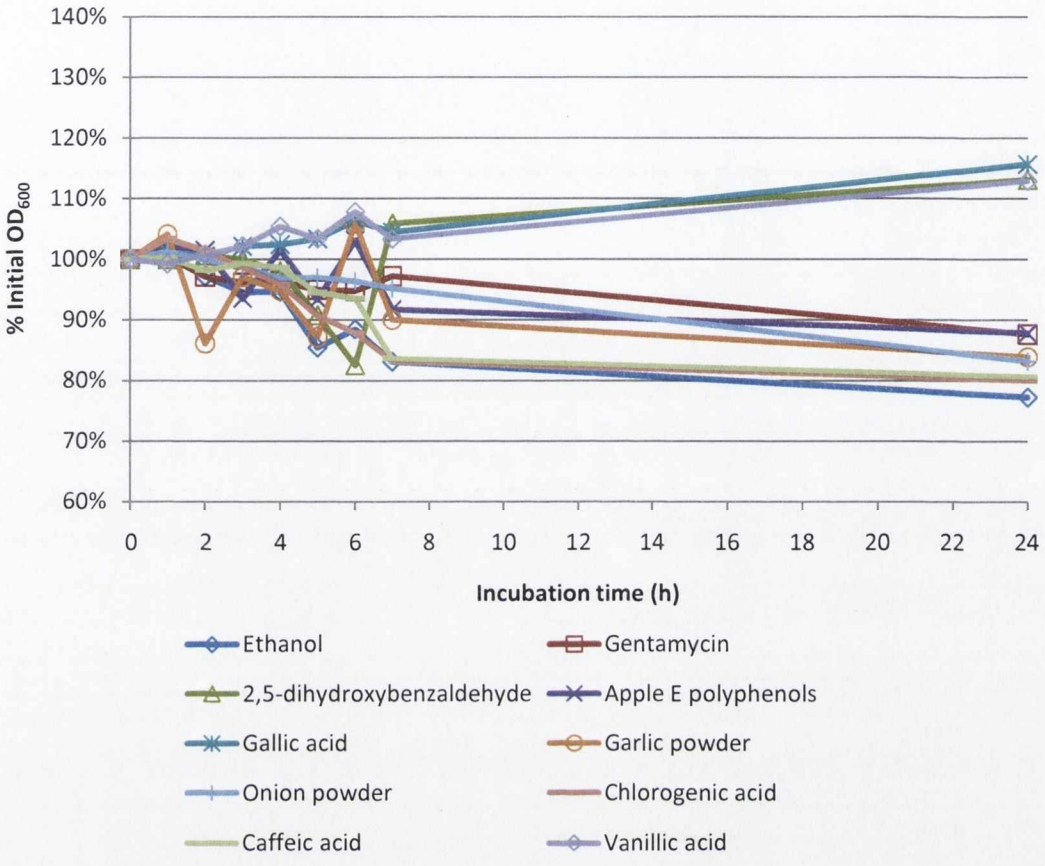

Figure 5.1.b Effect of naturally occurring compounds (powders) that have showed in previous study different degrees of bactericidal effect on *E. coli* culture as compared to gentamycin (positive control), non-inhibitory compound (vanillic acid), and non-inhibitory concentration of ethanol 1% (vol/vol),. All compounds were tested at 500 µg/ml except antibiotic gentamycin which was tested at 10 µg/ml.

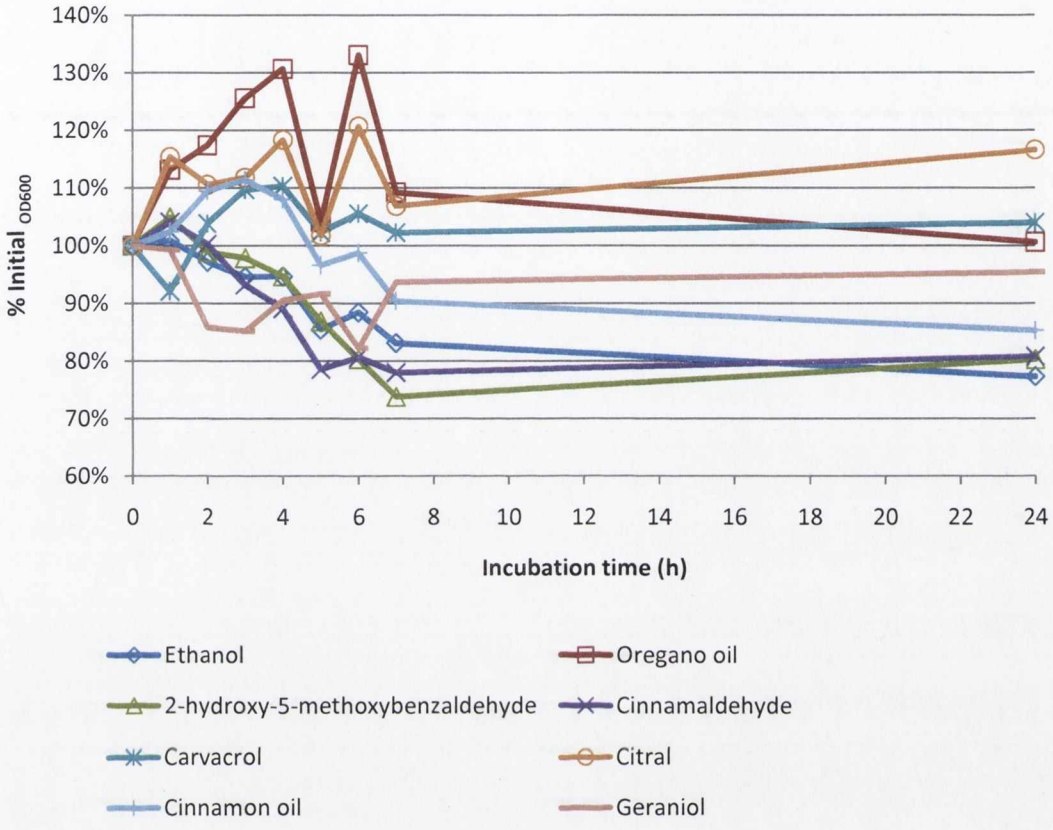

Figure 5.1.c Comparison of effect of naturally occurring compounds (oils) that showed different level of bactericidal effects on *E. coli* and non-inhibitory concentration of ethanol (1%, vol/vol) as the negative control onOD_{600} measurements of *E. coli* culture. All compounds were tested at 500 μg/ml.

5.3.2 Phosphate assay

The effect of the naturally occurring compound cinnamaldehyde on the phosphate leakage of *Map* is shown in figure 5.2.a. The results suggested that membrane permeability increased as indicated by the increase in extracellular phosphate levels in the presence of cinnamaldehyde and that such change was concentration and time dependent. Higher concentrations of phosphate were found in the culture supernatant when incubated with higher concentration of cinnamaldehyde for a longer period of time. Interestingly, at the MIC of cinnamaldehyde (24 μg/ml), there was no significance in the concentration of phosphate in the extracellular environment compared to the negative control ethanol. However, substantial leakage of phosphate occurred at concentrations higher than the MIC.

Figure 5.2.b shows the effects of four anti-*Map* naturally occurring compounds and two negative controls, non-active compound vanillic acid and ethanol on the phosphate leakage of *Map*. The results showed that membrane permeability increased in the presence of all of the naturally occurring compounds, including the non-active compound and such increase of membrane permeability was time dependent. The degrees of phosphate leakage varied among different compounds. Cinnamaldehyde caused the highest level of phosphate leakage, while 2,5-dihydroxybenzaldehyde resulted in the lowest level. Interesting, the level of phosphate leakage caused by non-active compound vanillic acid was higher than carvacrol and 2,5-dihydroxybenzaldehyde (both MIC = 74 μg/ml). The results suggested that leakage of phosphate may be as a cellular response to treatment with "foreign" compounds rather than being specific to compounds having antimicrobial properties.

The effects of seven antibacterial compounds and two negative controls (non-active compound vanillic acid and ethanol) on the phosphate leakage of *E. coli* are shown in Figure 5.2.c. The results showed that membrane permeability increased in the presence of all naturally occurring compounds, including the non-active compound and such increases were mainly time dependent (except a small variation in garlic powder at one time point). Apple E polyphenols caused the most severe leakage of phosphate while

gallic acid resulted in the lowest degree of leakage. There appeared to be no correlation between the antibacterial potency of the compounds and their capability of causing leakage of phosphate. The level of phosphate leakage caused by non-active compound vanillic acid was higher than or equal to two of the bactericidal compounds gallic acid and green tea polyphenols. Similar to the findings observed in *Map* cultures, these results suggested that leakage of phosphate is a non-specific cellular response to treatment with "foreign" compounds rather than being specific to compounds which have antimicrobial activities.

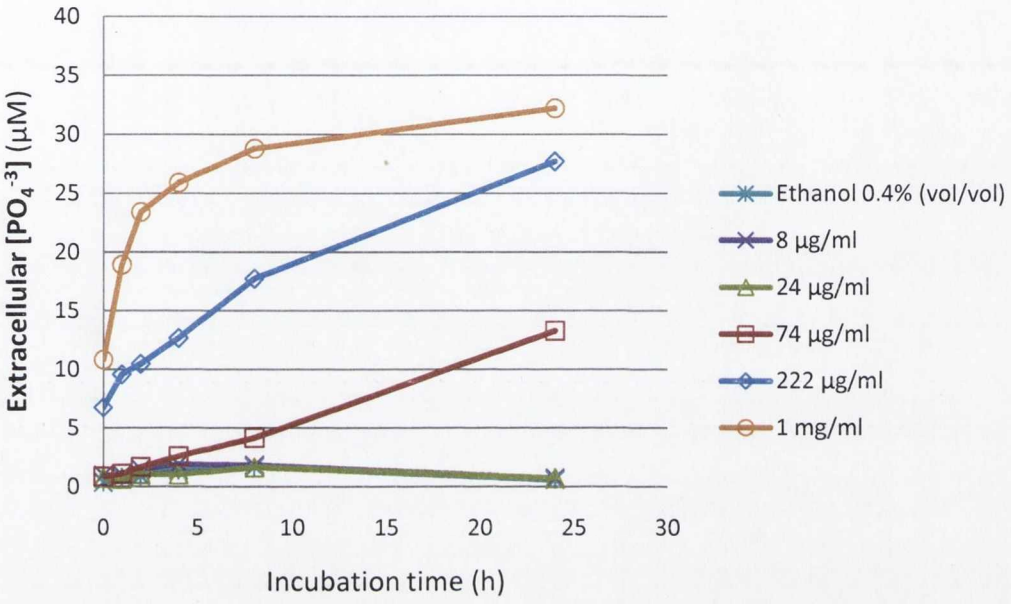

Figure 5.2.a - Extracellular levels of phosphate ion in aliquots of *Map* cultures incubated with various concentrations of cinnamaldehye (MIC24 µg/ml) and non-inhibitory concentration of ethanol (0.4%, vol/vol) as the negative control.

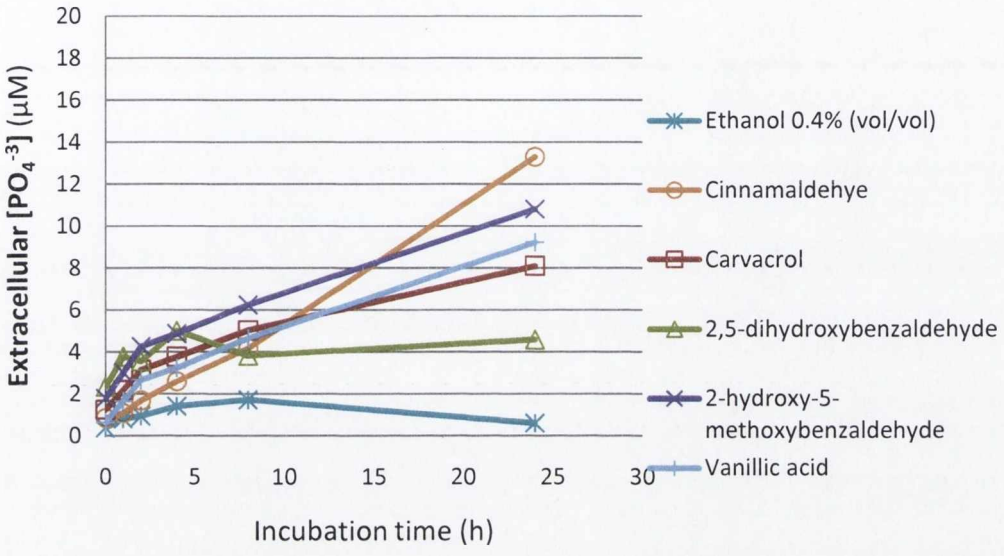

Figure 5.2.b Extracellular concentration of phosphate ion in cultures of *Map* incubated with different active compounds, non-inhibitory compound (vanillic acid) at 74 μg/ml, and non-inhibitory concentration of ethanol (0.4%, vol/vol).

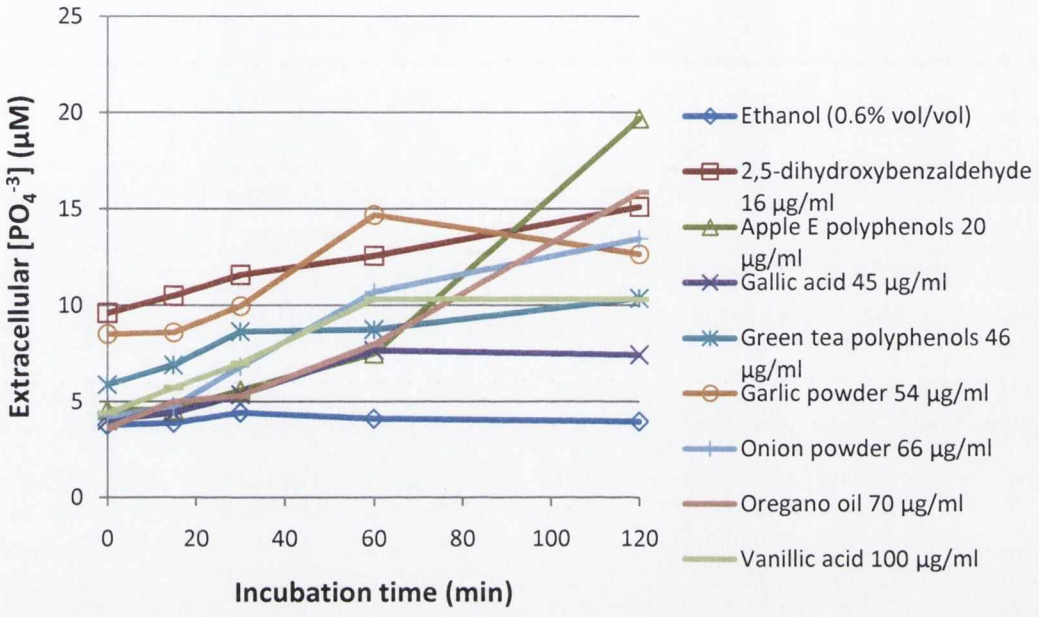

Figure 5.2.c Extracellular concentration of phosphate ion in aliquots of *E. coli* incubated with various active compounds at their MICs, non-inhibitory compound (vanillic acid) at 100 µg/ml, and non-inhibitory concentration of ethanol (0.6%, vol/vol).

5.3.3 Leakage of nucleic acid

The effects of the addition of the naturally occurring compound cinnamaldehyde on DNA leakage of *Map* at various concentrations are shown in figure 5.3.a. The results showed that DNA was found in extracellular environments when incubating *Map* with different concentrations of cinnamaldehyde (≥MIC). Higher DNA concentrations were obtained as the incubation time increasing. The most severe DNA leakage was observed at 74 μg/ml of cinnamaldehyde but not the highest concentration tested.

Figure 5.3.b shows the effect of the addition of naturally occurring compound vanillic acid (non-active) at various concentrations on the DNA leakage of *Map*. The results showed that DNA was found in extracellular environment when incubating *Map* with all concentrations of vanillic acid tested. Higher DNA concentrations were observed generally at the longest incubation time. Compared to cinnamaldehyde, the DNA concentration found in the extracellular environment was higher in the presence of vanillic acid.

Figure 5.3.c illustrates the effects of the four anti-*Map* naturally occurring compounds at their MICs and two negative controls on DNA leakage of *Map*. The results showed that higher DNA concentrations were observed generally at the longest incubation time, similar to the samples tested with various concentrations of cinnamaldehyde and vanillic acid. The results may indicate that mild leakage of DNA is a cellular response to treatment with naturally occurring compounds but not specific to the antimicrobial effect of the compounds and their effective concentrations.

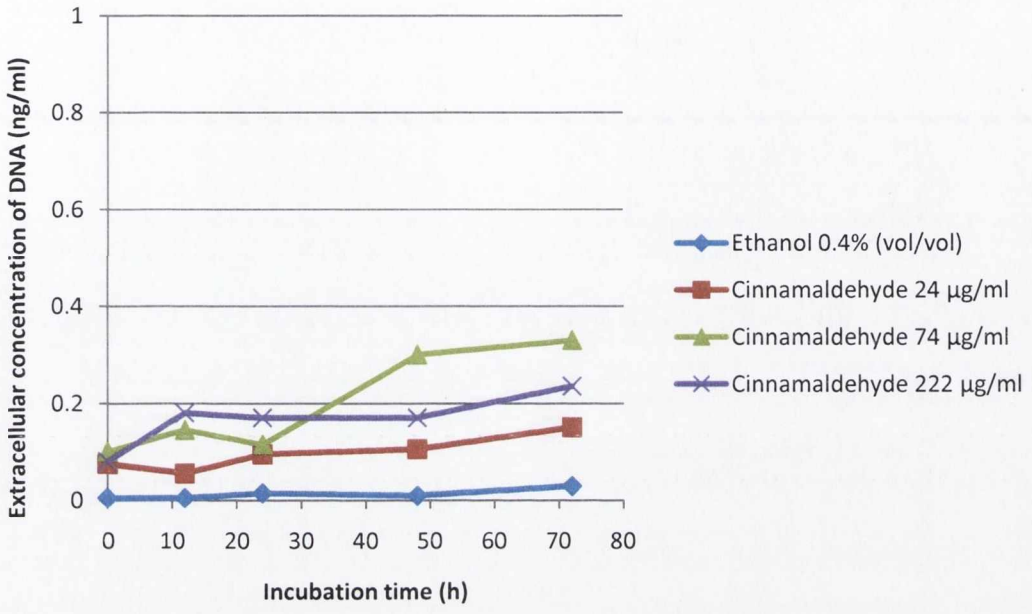

Figure 5.3.a Extracellular concentration of DNA in cultures of *Map* incubated with various concentrations of cinnamaldehye (MIC = 24 µg/ml) and non-inhibitory concentration of ethanol (0.4%, vol/vol) as the negative control.

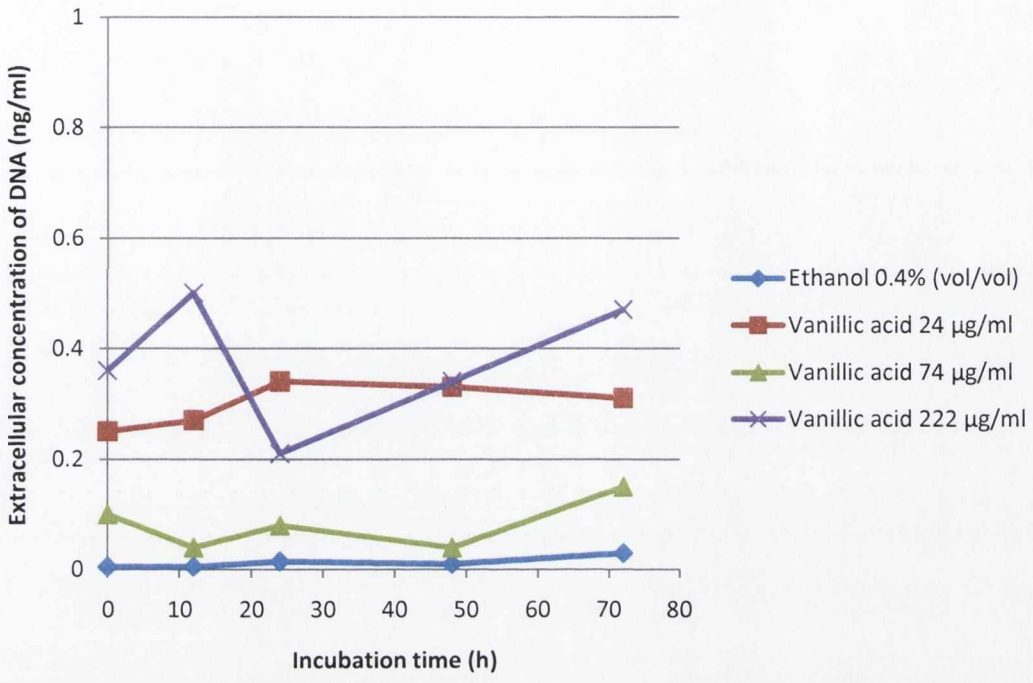

Figure 5.3.b Extracellular concentration of DNA in cultures of *Map* incubated with various concentrations of vanillic acid (non-inhibitory compound) and non-inhibitory concentration of ethanol (0.4%, vol/vol) as the negative control).

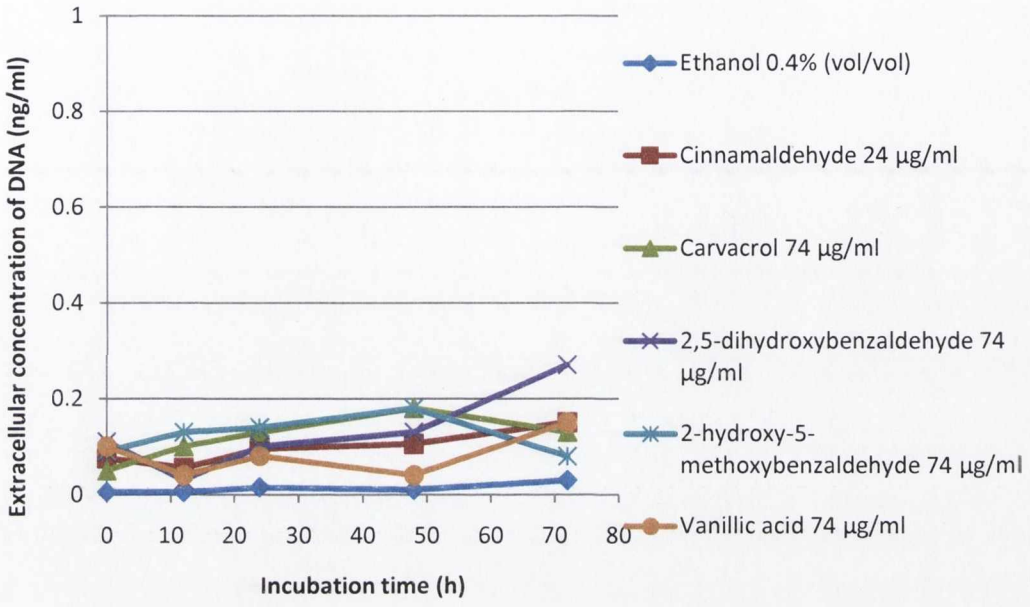

Figure 5.3.c Extracellular concentration of DNA in cultures of *Map* incubated with various active compounds at their MICs, non-inhibitory compound, vanillic acid at 74 µg/ml and non-inhibitory concentration of ethanol (0.4%, vol/vol).

5.3.4 Leakage of protein

Figure 5.4.b shows the effect of the addition of four anti-*Map* naturally occurring compounds at their MICs, non-active compound vanillic acid, and ethanol on the protein leakage of *Map*. The result showed that a maximum concentration of 2 μg/ml of protein was found in the extracellular environment but no increased protein levels were observed. As the effective range of the calibration curve (figure 5.4.a) was 1 – 10 μg/ml, comparing the amount of proteins detected in the samples to the calibration curve, the protein leakage was low and not obvious. Both cinnamaldehyde and 2-hydroxy-5-methoxybenzaldehyde showed an increase of protein (up to 50%) concentrations as incubation times increased. However, a decrease in protein levels was observed with carvacrol from 1.8 μg/ml to 0 μg/ml after 48 h. The negative controls of ethanol and non-active compound vanillic acid had the similar patterns but no trend observed.

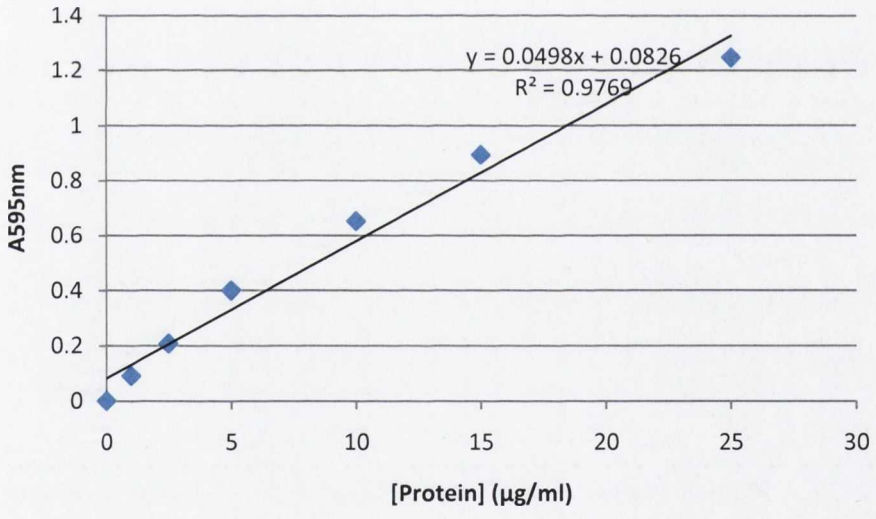

Figure 5.4.a Calibration curve of protein prepared by using BSA.

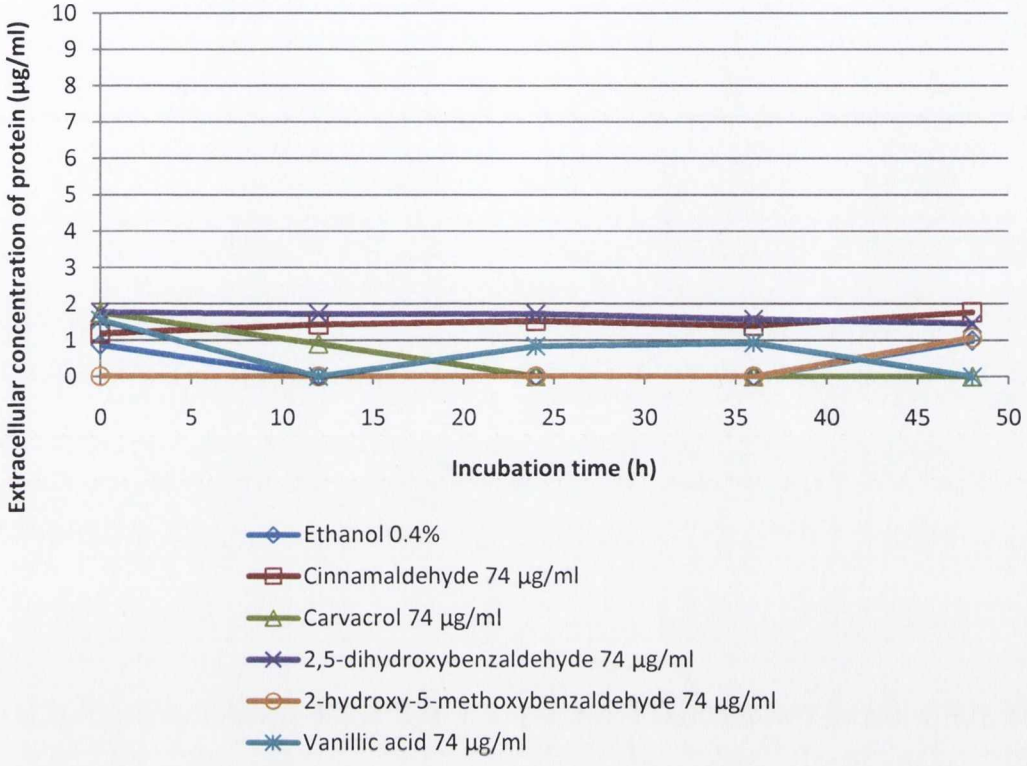

Figure 5.4.b Extracellular concentration of protein in cultures of *Map* incubated with various active compounds at their MICs, non-inhibitory compound, vanillic acid at 74 µg/ml, and non-inhibitory concentration of ethanol (0.4%, vol/vol).

5.3.5 ATP assay

In our study, the *Map* culture was subjected to starvation in order to deplete the intracellular ATP before the experiment. If bacterial cultures were analysed without starvation, the basal level of ATP in the cells might make it difficult to observe any decrease of intracellular ATP if the effect of the test compounds was merely inhibiting the synthesis of ATP rather than causing hydrolysis of ATP.

Figure 5.5.a shows the effects of six anti-*Map* naturally occurring compounds at 74 μg/ml and two negative controls, non-active compound 2,4,6-trihydroxybenzaldehyde and ethanol on the intracellular ATP concentration of *Map*, which had starved for 6 weeks. Intracellular ATP concentrations varied in all samples between times 0 - 8h. However, when considering incubation periods between 8 – 24 h, non-active compound 2,4,6-trihydroxybenzaldehyde, control ethanol, active compounds oregano oil and its active ingredient carvacrol showed an increase of ATP concentration, while active compounds cinnamon oil and its active ingredient cinnamaldehyde, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde showed a decrease of ATP concentration. However, the decrease of intracellular ATP concentration in the presence of 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde was small and not statistically significant ($p > 0.05$).

Figure 5.5.b illustrates the effect of the addition of six anti-*Map* naturally occurring compounds at 74 μg/ml and two negative controls, non-active compound 2,4,6-trihydroxybenzaldehyde and ethanol on the extracellular ATP concentration of *Map*, which had starved for 6 weeks. The results showed that extracellular ATP concentration was very low in all the samples treated with both active compounds and negative controls throughout the incubation period.

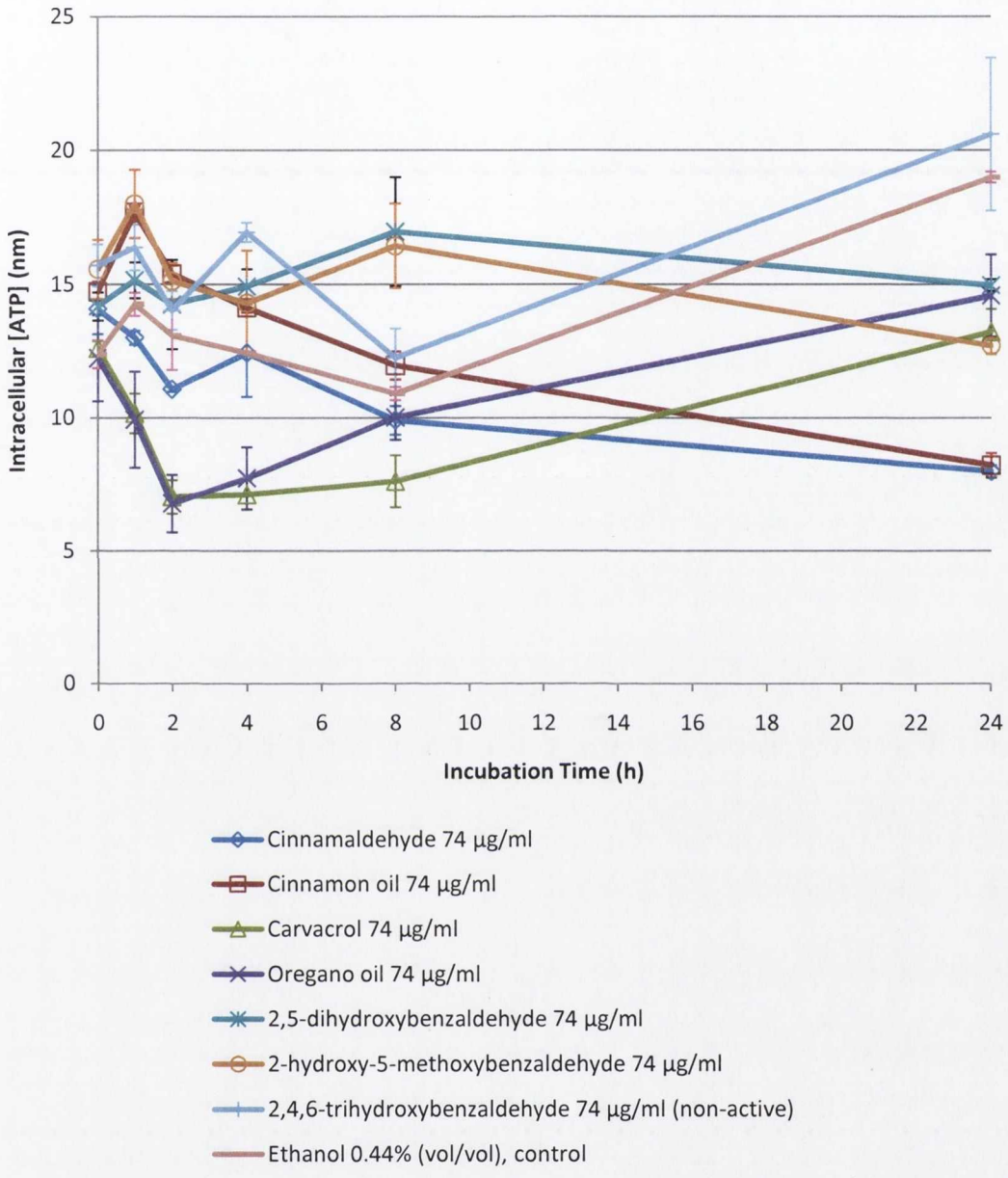

Figure 5.5.a Intracellular concentration of ATP in cultures of *Map* (subjected to 6-week starvation prior to experiment) incubated with various active compounds, non-inhibitory compound, 2,4,6-trihydroxybenzaldehyde at 74 µg/ml, and non-inhibitory concentration of ethanol (0.44%, vol/vol). Vertical bars indicate standard error of the mean (SEM).

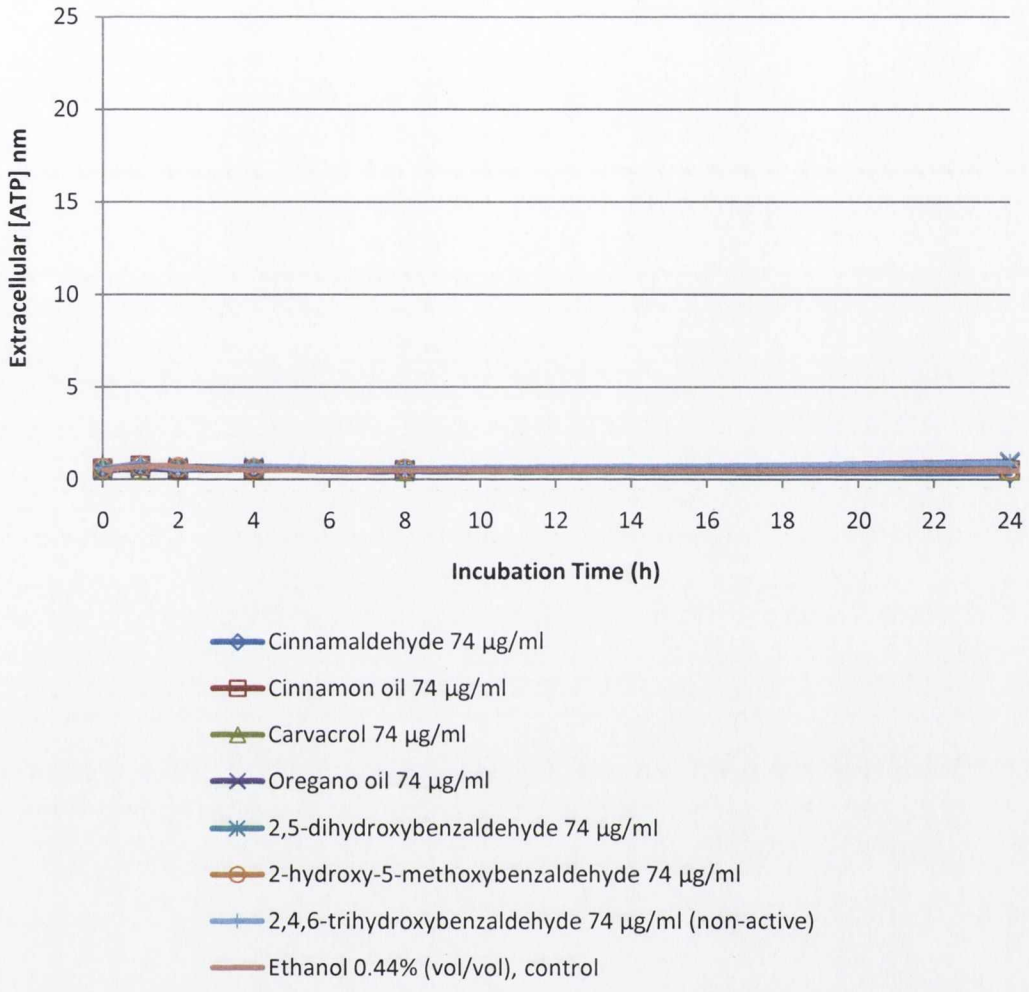

Figure 5.5.b Extracellular concentration of ATP in cultures of *Map* (subjected to 6-week starvation prior to experiment) incubated with various active compounds, non-inhibitory compound, 2,4,6-trihydroxybenzaldehyde at 74 µg/ml, and non-inhibitory concentration of ethanol (0.44%, vol/vol).

5.3.6 Absorbance scans of culture supernatant after antimicrobial treatment

As shown in figure 5.6.a, a peak was observed and it reached the highest absorbance within one hour at 290 nm (A_{290}). The A_{290} reading represents the relative abundance of cinnamaldehyde in the sample, which decreased from about 2.75 to 2.0 as the time of incubation increased during the 4-day incubation period. It indicated that the quantity of cinnamaldehyde in the sample was decreasing which might due to adsorption of cinnamaldehyde into the plastic sample container during incubation, the longer the incubation time, the more the cinnamaldehyde molecules adsorbed to the sample container, thus the reading was decreasing as a function of time.

The A_{290} reading decreased as the time of incubation increased, which indicates that the quantity of cinnamaldehyde was decreasing (figure 5.6.b). It might again due to adsorption to the plastic container. Apart from that, when comparing the time 0 min of this supernatant to the corresponding blank, the A_{290} reading is approximately 0.5 lower in the supernatant, the reason might due to the adsorption of cinnamaldehyde to the *Map* cell surface or uptake of cinnamaldehyde into the *Map* cultures. Furthermore, a new peak, which had the highest absorbance at about 250 nm appeared after one day of incubation. The new peak could either be a product transformed by *Map* from cinnamaldehyde, or an intracellular constituent leaked out from the *Map* cells.

Indicated in figure 5.6.c, the highest absorbance was observed at about 270 nm (A_{270}). The A_{270} reading represents the relative abundance of carvacrol in the sample, which decreased from about 0.58 to 0.48 as the time of incubation increased during the 4-day incubation period. Similarly, the absorbance scan of cinnamaldehyde in water, suggested that the quantity of carvacrol in the sample was decreasing, which might due to adsorption of carvacrol to the plastic sample container during incubation. However, the decrease was much smaller compared to cinnamaldehyde.

As shown in figure 5.6.d, the A_{270} reading decreased as the time of incubation increased, which indicated that the quantity of carvacrol was decreasing. Similar to the result obtained for cinnamaldehyde, it might be again due to the adsorption to the plastic

container and/or adsorption and uptake to the *Map* cultures. However, different from the observation for cinnamaldehyde, the decrease in absorbance reading in culture supernatant treated with carvacrol was much smaller and no additional peak was observed throughout the incubation period.

In figure 5.6.e, there was no distinct peak observed from the scan but the absorbance reading was slightly higher at A_{230}. There was no significant difference between the absorbance obtained at different time intervals. Similar to the supernatant incubated with carvacrol, no new peak was observed throughout the incubation period.

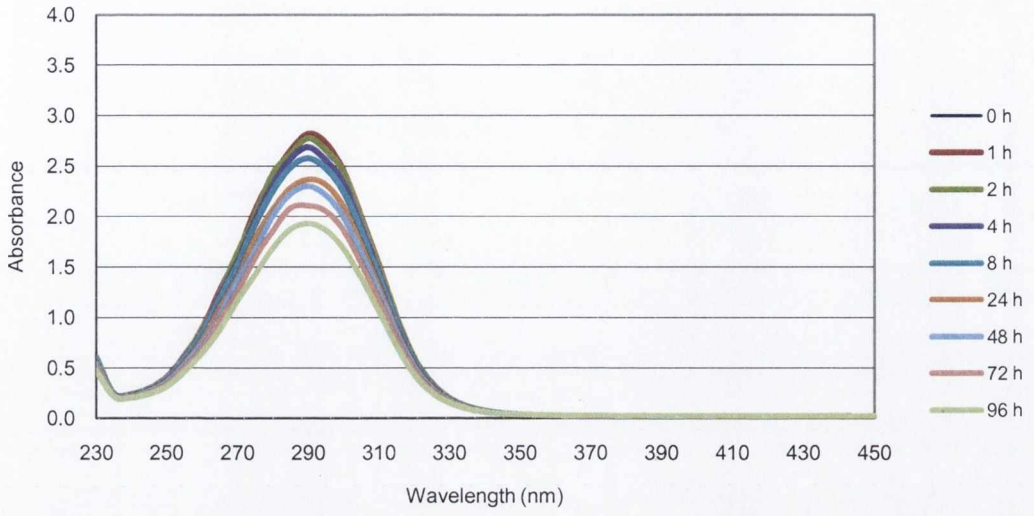

Figure 5.6.a Absorbance scan from 230 to 450 nm of 24 µg/ml cinnamaldehyde in sterile reverse osmosis water incubated at 37°C.

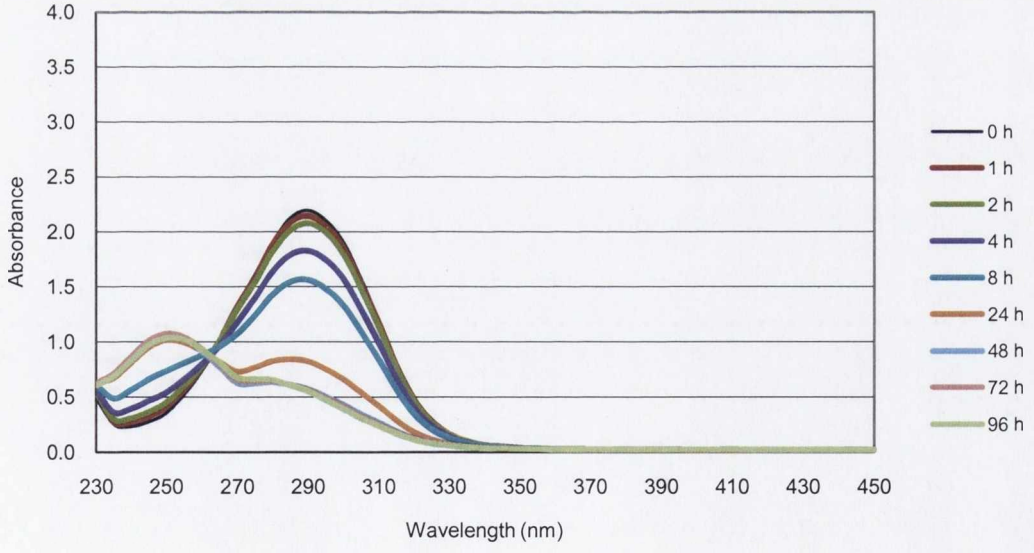

Figure 5.6.b Absorbance scan from 230 to 450 nm of supernatant of *Map* cultures incubated with 24 µg/ml cinnamaldehyde (the MIC that inhibits the growth of *Map* in broth medium) in sterile reverse osmosis water.

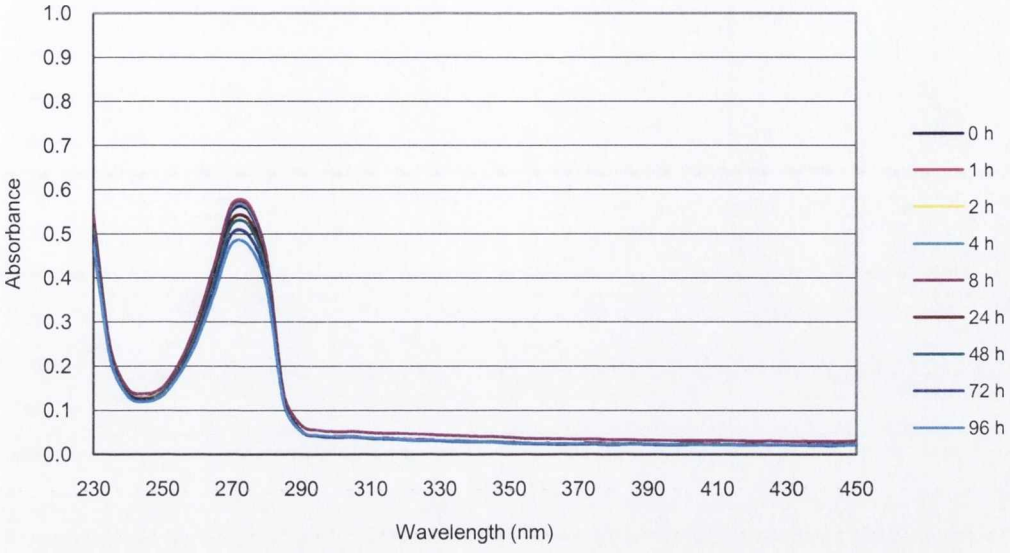

Figure 5.6.c Absorbance scan from 230 to 450 nm of 74 µg/ml carvacrol in sterile reverse osmosis water incubated at 37°C.

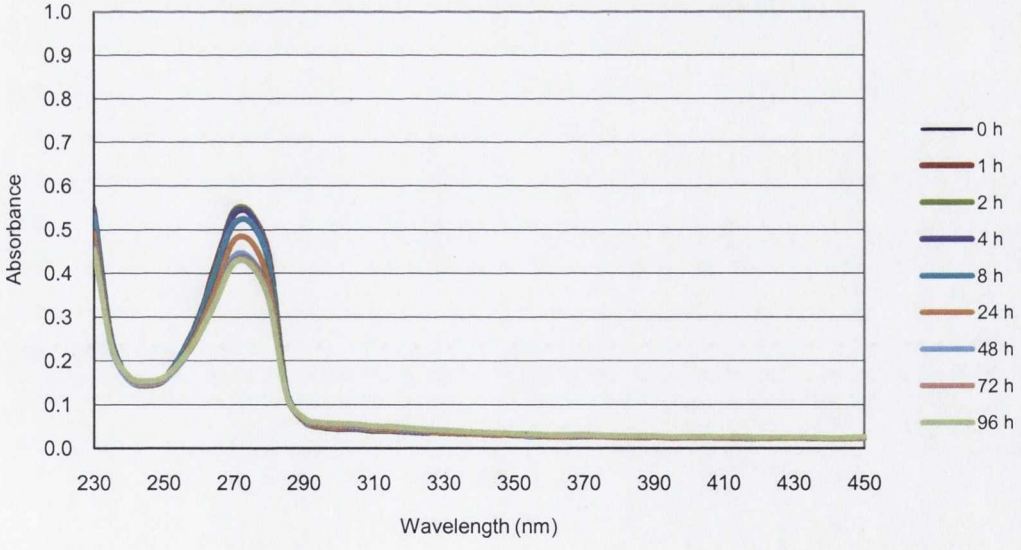

Figure 5.6.d Absorbance scan from 230 to 450 nm of supernatant of *Map* cultures incubated with 74 µg/ml carvacrol (the MIC that inhibits the growth of *Map* in broth medium) in sterile reverse osmosis water.

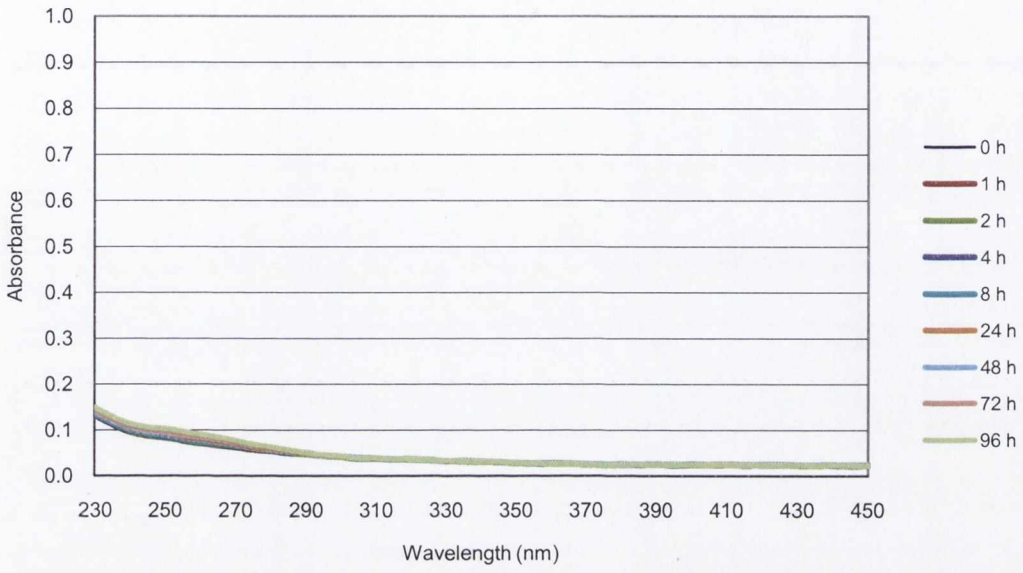

Figure 5.6.e Absorbance scan from 230 to 450 nm of supernatant of *Map* cultures incubated with negative control ethanol at non-inhibitory concentration 0.4% (vol/vol) in sterile reverse osmosis water.

5.4 Discussion

This study describes the cellular responses of animal pathogen *Map* and surrogate non-pathogenic *E. coli* in the presence of naturally occurring compounds, in terms of the level of cell autolysis, leakages of different intracellular constituents, and measurement of the internal ATP pool for gaining further insight into the possible modes of action of the naturally occurring compounds. Gustafson et al. (1998) demonstrated that tea tree oil caused autolysis in *E. coli* cultures in a time dependent manner. However, in our experiments none of the naturally occurring compounds showed autolysis on *Map* and *E. coli*. Our results suggested that whole cell autolysis was not the mode of action of these naturally occurring compounds tested against *Map* and *E. coli*.

The result of leakage of phosphate ion obtained from our study is similar to the study performed by Lambert et al. (2001). We observed that all naturally occurring compounds caused leakage of phosphate ion and such leakage was concentration and time dependent, however, this phenomenon was not only limited to the compounds that exert antimicrobial activity but also appeared in non-active compounds. The non-active compound vanillic acid had shown higher degree of phosphate leakage than two of the active compounds, carvacrol and 2,5-dihydroxybenzaldehyde at the same concentration. Lambert et al. (2001) demonstrated that oregano essential oil caused leakage of phosphate ion in both Gram positive organism *S. aureus* and Gram negative organism, *P. aeruginosa*, however, supplementary controls were not performed thus it is not known if the leakage observed from the two organisms was specific to oregano essential oil only. Our results showed that all naturally occurring compounds, including both active and non-active compounds caused certain stress on bacterial cultures, which is indicated by the increase in the level of phosphate ions found in the extracellular environment. However, such leakage stress was not specific or severe enough to cause growth inhibition or cell death, as the non-active compound, vanillic acid also caused a relatively severe leakage of phosphate at 74 μg/ml, thus leakage of phosphate ions was not considered as one of the mode of action for naturally occurring compounds tested against *Map* and *E. coli* but a cellular response.

Je and Kim (2006) demonstrated that chitosan derivatives caused leakage of intracellular components, e.g. DNA, which showed absorbance at 260 nm (A_{260}). The severity of leakage of such intracellular components was indicated by the increase in the reading of A_{260} only; while we expressed our results in terms of concentration of DNA in the supernatant samples. A relatively low DNA concentration was observed in all samples, the highest concentration obtained was below 0.5 ng/ml. The DNA leakages observed in our results were generally time dependent, however, it was not specific to the antimicrobial activity as results showed that the highest DNA concentration was obtained from *Map* cells treated with vanillic acid, a non-active compound to *Map*. The DNA leakage was also not specific to the concentration of compounds tested, for example, the highest DNA leakage (0.33 ng/ml) obtained from treatment with cinnamaldehyde was at 74 μg/ml rather than 222 μg/ml. Thus we can conclude that leakage of DNA was not the mode of action of the naturally occurring compounds tested against *Map*.

Kwon et al. (2003) reported a similar study on protein leakage using cinnamic aldehyde on *S. aureus* and *B. cereus*, both Gram-positive coccus. It was found the protein leakage level was significantly higher (~ 3 μg/ml) in the supernatant of *S. aureus* compared to the protein level of 0.7 μg/ml in *B. cereus*, after incubation with cinnamic aldehyde at MICs. They suggested that there are different mechanisms for the inactivation of the bacterial cells between the two organisms. In the present study, a lower protein concentration (<2 μg/ml) was detected but no increased protein levels were observed. The presence of protein in extracellular environment might due to a small portion of cells in the aliquot being weaker and more susceptible to foreign compounds and ethanol treatment, though the concentration of ethanol was kept low. Thus, we can conclude that leakage of protein was not the mode of action of the naturally occurring compounds tested against *Map*.

Gill and Holley (2004) had carried out two sets of experiments using starved bacterial cultures, firstly, ATP was measured in a sample of starved bacterial culture treated with test compounds first, then followed with glucose 5 min later; secondly, ATP was

measured in sample of starved bacterial culture added with glucose first, then followed with test compounds 5 min later. They found that cinnamaldehyde inhibited ATP synthesis in *Listeria monocytogenes* when added prior to glucose and rapidly declined the intracellular ATP when added after glucose. Our study has shown that the treatment of *Map* cultures, which had been starved for 6 weeks with anti-*Map* compounds, cinnamon oil and its constituent cinnamaldehyde led to a decrease in intracellular ATP concentration during the incubation period between 8 – 24 h, whilst an increase of intracellular ATP concentration was observed in *Map* culture treated with active compounds such as oregano oil, its constituent carvacrol and negative controls during the same incubation period. However, no significant effect of 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde on intracellular ATP concentration was observed ($p > 0.05$).

When considering the whole incubation period, cinnamaldehyde, the most potent compound, showed a rapid decrease of intracellular ATP concentration, that might due to a reduction in the rate of ATP synthesis or an increase in ATP hydrolysis. Cinnamon oil, which has the same MIC (24 μg/ml) as cinnamaldehyde showed an initial increase of intracellular ATP concentration, before the intracellular ATP concentration declined. Such a change might be due to a slower action of cinnamon oil compared to cinnamaldehyde, the active constituent. Assuming cinnamaldehyde is the only active ingredient in cinnamon oil, thus it might explain when treating *Map* with 74 μg/ml of cinnamaldehyde, the effect was quicker than treating *Map* with 74 μg/ml of cinnamon oil, which contains other substances that may not as effective as cinnamaldehyde.

Oregano oil and its active constituent carvacrol showed an initial decrease of intracellular ATP concentration, before the intracellular ATP concentration raised again. It might be because oregano oil and carvacrol can exert their antimicrobial effect by means of stressing the *Map* initially; however, *Map* was able to overcome such an attack and to decrease the effect to some extent, thus ATP synthesis might be resumed or ATP hydrolysis might slow down as result of a self-defending mechanism. However, their

final intracellular ATP concentrations were still low compared to the two negative controls.

2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde showed fluctuating intracellular ATP concentrations throughout the experiment. At the last time point (24 h), the intracellular ATP concentration in the presence of 2-hydroxy-5-methoxybenzaldehyde was slightly lower than in the presence of 2,5-dihydroxybenzaldehyde. However, their effect on intracellular ATP concentration throughout the incubation period was not significant ($p > 0.05$). Their final intracellular ATP concentrations were similar to those observed in oregano oil and carvacrol at their MIC level of 74 μg/ml. The results suggested that cinnamaldehye may have a different mode of antimicrobial action to *Map* cells compared to carvacrol, 2-hydroxy-5-methoxybenzaldehyde and 2,5-dihydroxybenzaldehyde.

ATP was found in the extracellular environment in all of the samples including both subjected to anti-*Map* compounds and negative controls. The concentration was however very low, steady against time and did not increase proportionally to the decrease of intracellular ATP concentration. Thus, it can be concluded that naturally occurring compounds tested did not increase the membrane permeability for ATP. The presence of ATP in the extracellular environment might be due to a small portion of cells in the aliquot were weaker and more susceptible to foreign compounds and ethanol treatment, though the percentage of ethanol was low, 0.4% (vol/vol). Another possibility would be the centrifugation was not effective enough to separate the *Map* culture from the supernatant completely, a small portion of cells was still present in the supernatant, thus the extracellular ATP found in the samples was contributed from that portion of cells.

Interestingly, Ultee et al. (1999) demonstrated the effect of carvacrol on both intracellular ATP and extracellular ATP of Gram positive bacterium, *B. cereus* and found that carvacrol caused rapid (within 14 min of addition of carvacrol) depletion of intracellular ATP, with small amount of ATP observed in the extracellular environment, which was not in proportion to the depletion of intracellular ATP. Such depletion might

be caused by rapid hydrolysis of ATP or inhibition of ATP synthesis. In a separate study, Helander et al. (1998) found that carvacrol caused a gradual decrease in the amount of intracellular ATP, with slight but significant leakage of ATP to the extracellular environment in *E. coli* culture, it indicated that the membrane had been damaged to a level that was permeable to ATP. However in our study carvacrol did not deplete the intracellular ATP pool in *Map*, which is also a Gram positive bacterium. Referring to the same study performed by Helander et al. (1998) mentioned previously, cinnamaldehyde was also tested and showed strong inhibition on growth of *E. coli* but did not deplete its intracellular ATP pool and in contrast to our study, cinnamaldehyde showed not only the strongest inhibition on *Map* growth but also depleted its intracellular ATP pool of *Map*. Comparing the studies performed by Ultee et al. (1999) and the present study, the difference on how bacterial cells responded in the presence of carvacrol might be explained by the differences in the cell membrane compositions, though *B. cereus* and *Map* are both Gram positive organisms, the cell membrane compositions are different, *Mycobacteria* are well known for their thick, waxy and hydrophobic cell wall, which is very rich in mycolic acid. These imply that natural essential oils could interact differently with different bacteria depending on their cell membrane compositions and cause different interruptions in metabolic activities.

Sikkema et al., (1995) suggested that the toxicity of a compound is highly related to its ability to disturb the hydrophobic interactions between the lipids and proteins in the cell membrane, thus decline the membrane integrity. It was believed that hydrophobic oil compounds interact with the bacterial cell membrane, change the membrane structure, stability and thus permeability to certain intracellular constituents. As suggested by Ultee et al. (1999), the consequence of leakage of essential ions could lead to inhibition of enzymes, loss of turgor pressure and affect different cellular processes and metabolic activities, which are vital for viability of cells.

The absorbance scan of the supernatant of *Map* showed that the naturally occurring compound, cinnamaldehyde at MIC, 24 μg/ml resulted in the appearance of a new peak at about 250 nm after one day of incubation. The new peak could either be a product

transformed by *Map* from cinnamaldehyde during the incubation, or an intracellular constituent leakage from the *Map* cells. As the peak showed the highest absorbance within the UV region (250 nm), it is possibly an indication of protein leakage from the *Map* cells, though this was not in agreement with the result obtained from the extracellular protein assay in the previous section. The appearance of such a new peak was specific to cinnamaldehyde only but not to other anti-*Map* naturally occurring compounds or the negative control ethanol. Experiments were carried out to try and identify this peak, including analysis of the sample using QToF LC/MS system and NMR spectroscopy; however, those attempts were not able to identify the peak. Further experiments, e.g. GC/MS might carry out for its identification (Floyd S. personal communication, 24 August, 2009).

To conclude, the cellular responses of animal pathogen *Map* and *E. coli* in the presence of naturally occurring compounds were studied. Naturally occurring compounds tested did not cause cell autolysis in *Map* and *E. coli* cultures and appreciable leakages of DNA and soluble protein in *Map* cultures. They caused leakage of phosphate ions in *Map* and *E. coli* cultures in relation to time and concentration of the test compounds, however, the severity of phosphate leakage was not specific to the antimicrobial activity of the compounds. Naturally occurring compounds, cinnamon oil, its constituent cinnamaldehyde and 2-hydroxy-5-methoxybenzaldehyde caused a decline in intracellular ATP concentration but not leakage of ATP to extracellular environment. Cinnamaldehyde might have caused leakage of cellular constituent or transformation of new product in *Map*.

6 Determination of Possible Modes of Antimicrobial Action of Naturally Occurring Compounds using Model Membranes

6.1 Introduction

Naturally occurring compounds have been known for their antimicrobial activity since antiquity. Due to the concern arising from the development of antibiotic resistant bacteria associated with the frequent use of antibiotics, increasing efforts have been placed into exploring natural resources for novel antimicrobial discovery to aid in new drug development for both human and veterinary medicine. The naturally occurring compounds selected in this study are mainly essential oils and their isolated constituents; they are secondary plant metabolites, including phenolic compounds, polyphenols and benzaldehydes.

Recent studies have focused on screening bioactive compounds using antimicrobial assay coupling with investigation of the modes of action of the identified active components (Gill and Holley, 2004, Helander et al., 1998, Friedman et al., 2003). In addition, studies of the structure and function relationship of the active components can help in identifying the important functional group of the component responsible for the antimicrobial activity, which might be beneficial to maximise the potencies of the existing antimicrobials (Epand and Epand, 2009a, Epand and Epand, 2009b). It is generally accepted that the naturally occurring compounds target the bacterial cell membrane to exert their antimicrobial effects owing to their hydrophobic-hydrophilic nature, however, the exact molecular interaction between naturally occurring compounds and membrane remains unknown to date (Burt, 2004).

In microbiological experiments studies have focused on the investigation of the cellular responses of bacteria subjected to non-lethal bacterial treatment, e.g. change of membrane integrity which could be indicated by leakage of intracellular constituents to the extracellular environment; change of relativity of membrane potential (Ultee et al.,

1999), intracellular pH (Ultee et al., 1999) and ability of ATP synthesis (Ultee et al., 1999, Gill and Holley, 2004, Helander et al., 1998). All these experiments involve the use of complex living bacterial culture. A new interpretation of the role played by membrane lipids in many cellular physiological processes has recently gained increasing attractions (Mecheri et al., 2004, Giordani et al., 2006, Dennison et al., 2009).

Monolayer is a technique studying the thermodynamic properties of a model membrane system at the water-air interface (Maget-Dana, 1999). Despite the fact that the biological membrane is composed of bilayer, monolayer technique has been employed to study the characteristics of lipid systems mimicking mammalian membrane and bacterial membrane (Brockman, 1999). As the biological membrane is a complex system and contains many variables, it can make further analysis and interpretation of results difficult.

Recently the role of membrane lipids in cellular function has been revised intensively, the classic fluid mosaic model has been enriched by the new lipid rafts model (Giordani et al., 2006). It has been found that lipid rafts or domains also exist in bacterial membranes but the knowledge of these compositions is much lagged in development (Epand and Epand, 2009a, Epand and Epand, 2009b).

The monolayers chosen in this study were composed of DPPE and DOPE, which are zwitterionic lipids; and DPPG, DOPG and cardiolipin, which are anionic lipids, as they are the most dominated phospholipid found in bacteria. In general, Gram-negative bacteria are rich in zwitterionic phospholipid in their membrane and also contain anionic phospholipid, while Gram-positive bacteria contain predominantly anioin lipid (Epand and Epand, 2009a, Epand and Epand, 2009b).

To our knowledge, this study represents the first evaluation of the activity of naturally-occurring compounds against monolayers composed of phospholipids dominated bacterial membrane. Lipid monolayers were used to study the ability of the naturally occurring compounds to interact with models mimicking bacterial membranes. The

monolayer technique allowed us to focus solely on the influence of phase behaviour on the interactions between natural antimicrobials and phospholipids, by recording surface-area isotherms and calculating from these data the interfacial elastic moduli of area compressibility (C_S^{-1}), providing an indicator for changes in the structure of the monolayer. Information on the electric properties of the monolayer was obtained from the measurement of surface dipole potential.

6.2 Materials and methods

6.2.1 Chemicals.

1,1',2,2'-tetratetradecanoyl cardiolipin sodium salt (CL), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt (DOPG), 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dihexadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) sodium salt (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Stock solutions of the lipids (5 mg/ml) were prepared in chloroform (Sigma, UK) and stored at -20°C. Reverse osmosis water (Milli Q water system; Millipore, France) was freshly obtained and used in all experiments.

The following naturally occurring compounds were originally obtained from Sigma (St. Louis, MO, USA): 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, 2-hydroxy-5-methoxybenzaldehyde, carvacrol, *trans*-cinnamaldehyde and geraniol. The purity levels of these compounds ranged from 95 to 99.9% according to the manufacturer. Apple E (concentrated apple polyphenols) was originally obtained from Apple Poly LLC (Morrill, NE, USA); the purity level was approximately 82%. Green tea polyphenols was originally obtained from LKT Laboratories, Inc. (St. Paul, MN, USA); the purity level was not specified. The above compounds were gifts from Dr Mendel Friedma, Western Regional Research Center, U.S. Department of Agriculture, Albany, California, USA. The naturally occurring compounds were prepared as stock solutions in reverse osmosis water for powder compounds (100 mg/ml) and chloroform for oil compounds (500 mg/ml). The stocks of powder compounds were freshly prepared before each experiment and used on the same day. The stocks of oil compounds were stored at -20°C. The compounds were tested at concentrations closed to the minimum inhibitory concentration (MIC) determined in the antibacterial assays in the previous studies, which were between 50 to 74 μg/ml for powder compound and as for oil compound, 1 μl of the above stock was deposited onto the surface of the subphase, the concentration could not be determined as the oil compounds were surface active and no shaking or stirring was employed to facilitate mixing.

6.2.2 Monolayer measurements

A Langmuir trough (Precision Plus) (μTrough XL; Kibron, Helsinki, Finland) equipped with a computer-controlled microbalance (Kibron) and MicroSpot (Kibron) was used to measure surface pressure-area (π-A) and surface potential-area (Δψ-A) isotherms respectively, using the embedded features of the control software (FilmWare 3.61; Kibron). The total surface area of the trough was 227.15 cm^2, and the volume of the subphase was approximately 100 ml.

The lipids prepared in chloroform were deposited onto the water surface (pure water with different natural compounds in subphase) interface using a 5-μl Hamilton micro-syringe (Supelco, Bellefonte, PA, USA). The selection of subphases was base on the antimicrobial assays performed in previous studies.

After 10 min equilibrium (to ensure evaporation of the solvent), film compression started by moving the two barriers symmetrically. In all measurements, the compression rate was 21.157 $Å^2$/chain/min to allow for the reorientation and relaxation of the lipids during the compression.

Surface pressure (π) was measured with the accuracy of ±0.1 mN/m using a metal wire probe (Kibron) linking with a high precision microbalance connected to a computer and is defined as follows:

$$\pi = \gamma_0 - \gamma$$

where γ_0 is the surface tension of the water and γ is the value for surface tension in the presence of a lipid monolayer.

Surface potential (ψ) was measured with the accuracy of ±0.1 mV using the vibrating plate method (MicroSpot; Kibron). All isotherms were recorded at 23 °C. The subphase temperature was controlled thermostatically to within 0.1 °C by a circulating water system (Grant Instruments (Cambridge) Ltd, Cambridgeshire, UK).

It has been reported that the oxidation of lipids in monolayers showed an observable extent only after ~30 to 40 min of air exposure (Benvegnu and McConnell, 1993, Sabatini et al., 2008), whereas these experiments were generally completed in less than 30 min, thus oxidation should not affect the results.

6.2.3 Analysis of isotherms

The value for monolayer isothermal compressibilities (C_S) for the indicated film compositions at the given surface pressure (π) was obtained from π -A data as follows:

$$C_S = (-1/A_\pi)\,(dA/d\,\pi)_T$$

Where A_π is the area per molecule at the indicated surface pressure π. To identify the phase transition points, we further analyzed the data in terms of the reciprocal isothermal compressibility (C_S^{-1}), which was performed by other researchers before (Smaby et al., 1996). Accordingly, the higher the value of the compressibility modulus C_S^{-1}; the lower the interfacial elasticity will be (Brockman et al., 1980).

6.2.4 Bacterial strains, growth condition and total lipids extraction from bacterial cells.

Map strain NCTC 8578, a bovine isolate obtained from the National Collection of Type Cultures, Colindale, London, was tested in this study. The strain was maintained and cultivated as described in Chapter 3 of this thesis.

The total lipid extraction was carried out as described by Bligh and Dyer (1959) with some modifications. Early stationary phase *Map* culture was de-clumped and centrifuged at 4,400 rpm for 30 min to pellet the cells. The pellet was then washed once with reverse osmosis water and resuspended in 1 ml of water and added with 3.75 ml of methanol-chloroform (2:1, vol/vol). The mixture was incubated at room temperature for 1 – 2 h with intermittent shaking. The mixture was then spun and the supernatant decanted into a fresh centrifuge tube; and the residue resuspended in 4.75 ml of methanol-chloroform-water (2:1:0.8, vol/vol), mixed and spun. Both portions of supernatant were combined in

one centrifuge tube and added with 2.5 ml each of chloroform and water, the mixture was spun. The lower chloroform phase was withdrawn carefully avoiding the water phase by using positive pressure/bubbling and ~90% of the chloroform was recovered to avoid recovery of water. The recovered lipid extract was stored at -20°C. Twenty five microliter of the extract was used in each measurement.

6.3 Results

6.3.1 Compression isotherms and compressibility modulus C_s^{-1}

The compression isotherms for DPPE monolayer deposited on different subphases were recorded (figure 6.1.a). In subphase containing pure water only, the lift-off value was approximately 47 $Å^2$ per molecule and increased sharply into the liquid-condensed (LC) phase up to around 25 $Å^2$ per molecule, at which monolayer collapses (collapse pressure was about 55 mN/m).

In contrast, isotherms of DPPE recorded on the subphase added with different naturally occurring antimicrobial compounds, showed the lift-off value varied from approximately 28 to 117 $Å^2$ per molecule and finally achieved the collapse pressure of approximately 40 to 55 mN/m. The addition of compounds to subphase changed the shape of the isotherms, which reflected modification of thermodynamic properties of the lipid monolayer, e.g. alteration of molecular area of DPPE molecule occupied in the monolayer, change of molecular packing effectiveness and change of membrane fluidity.

As shown in figure 6.1.a, when DPPE monolayer is compressed on subphase added with naturally occurring compounds 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde and apple E polyphenols at various concentrations (concentrations closed to the minimum inhibitory concentration MIC determined in antimicrobial assays in the previous study), the lift-off value dropped to about 28 to 33 $Å^2$ per molecule and then the monolayer collapsed at about 14 to 18 $Å^2$ per molecule (collapse pressure around 47 to 55 mN/m). The slope of the isotherm was steeper, which meant the phase transition from gas (G) state into the LC state was sharper than for pure water. This indicated a better packing effectiveness of DPPE molecules in the presence of those compounds.

When DPPE monolayer was compressed on subphase added with naturally occurring compounds green tea polyphenols, cinnamaldehyde, carvacrol and geraniol, the shape of the isotherm was greatly affected, these compounds increased the lift-off value up to

about 64 ~ 117 $Å^2$ per molecule and the monolayer collapsed at about 22 to 28 $Å^2$ per molecule (collapse pressure around 38 to 50 mN/m). The appearance of the liquid-expanded/liquid-condensed (LE-LC) region induced by these compounds was not observed in the monolayers compressed on other subphases.

2-hydroxy-5-methoxybenzaldehyde showed different effect on the isotherm of DPPE compared to other compounds, it did not alter the lift off value compared to water but decreased the collapse pressure to 47 mN/m at a smaller area of 23 $Å^2$ per molecule. The slope of the isotherm was flatter and did not induce the formation of liquid-expanded/liquid-condensed (LE-LC) region.

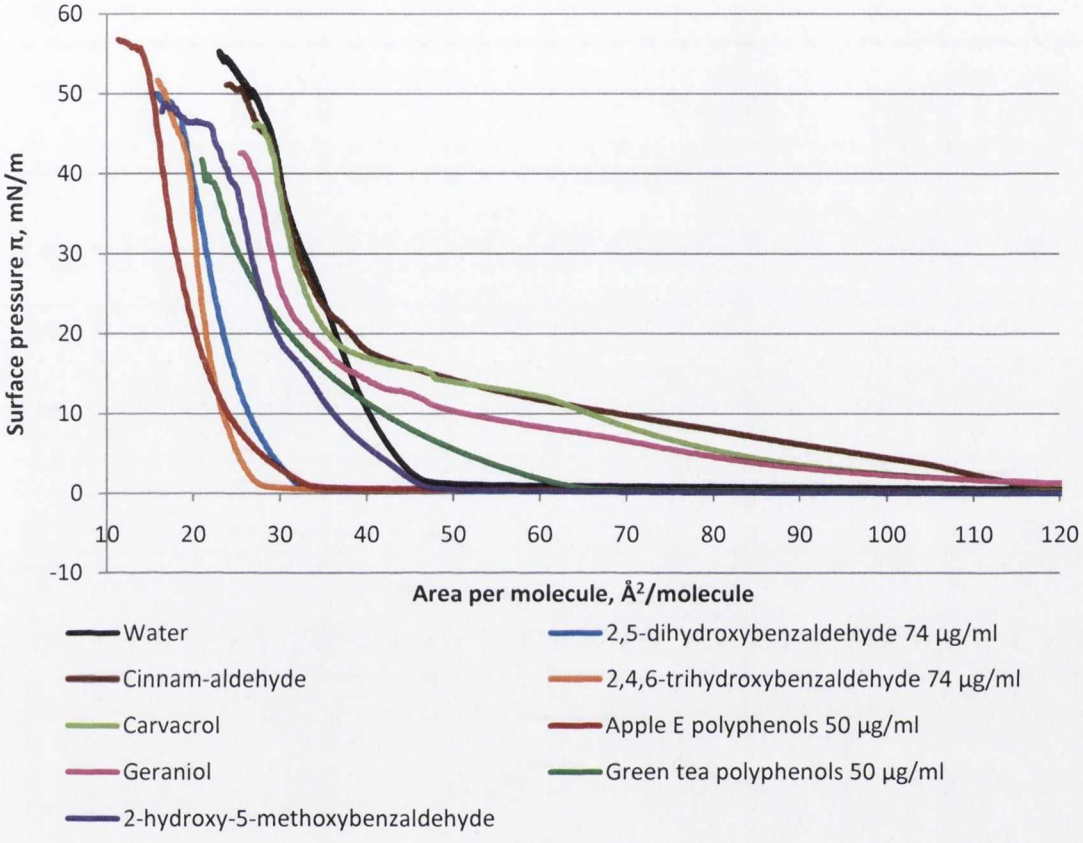

Figure 6.1.a The surface pressure-area (π-A) isotherms recorded for the monolayers formed by DPPE on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Analysis of the compression modulus for DPPE monolayer in the presence of different naturally occurring antimicrobial compounds is shown in figure 6.1.b. For DPPE monolayer compressed on subphase containing pure water only, two maximal values of C_S^{-1} (115 and 180 mN/m) were observed. This result was in agreement with those of Wydro and Witkowska (2009), as they also observed, during compression of DPPE monolayer, the phase transition between liquid condensed and solid state occurred.

The maximal value of the compression modulus for DPPE monolayer compressed on subphase containing 2,4,6-trihydroxybenzaldehyde was approximately 120 mN/m, which showed a slight rigidifying effect. For the remaining compounds, the maximal values of the compression modulus varied from approximately 78 to 110 mN/m. Such decreases in the maximal value of the compression modulus suggested a fluidizing effect may be caused by these compounds in the DPPE monolayer. The lower the maximal value of the compression modulus, the higher the fluidity of the monolayer (Wydro and Witkowska, 2009). Among these compounds, carvacrol exhibited the most significant fluidizing effect with the lowest the maximum compression modulus. All compounds tested had induced the formation of fluid pores between lipid rafts in the monolayer.

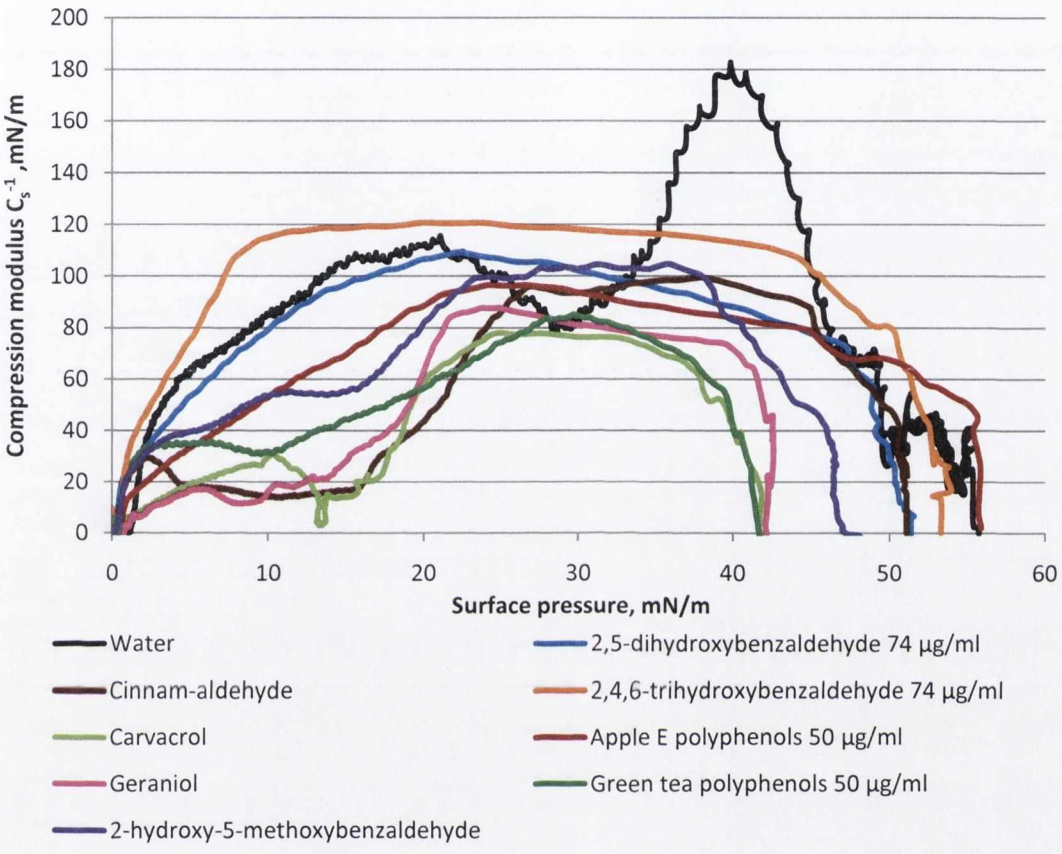

Figure 6.1.b The compression modulus (C_S^{-1}) values versus surface pressure (π) recorded for the monolayers formed by DPPE on subphases containing pure water with and without naturally occurring antimicrobial compounds.

The compression isotherms for DPPG monolayer deposited on subphases containing selected naturally occurring antimicrobial compounds were recorded and are shown (figure 6.2.a). In subphase containing pure water only, the lift-off value was approximately 90 $Å^2$ per molecule and increased gradually into the liquid-condensed (LC) phase up to around 55 $Å^2$ per molecule, at which monolayer collapsed (collapse pressure was about 49 mN/m).

In contrast, isotherms of DPPG deposited on subphase with naturally occurring antimicrobial compounds cinnamaldehyde, carvacrol and geraniol showed the lift-off values increased significantly ($p < 0.001$) to approximately 185 to 200 $Å^2$ per molecule and then the monolayer collapsed at about 43 to 53 $Å^2$ per molecule (collapse pressure around 38 to 43 mN/m), the slope of these isotherms was the flattest, which indicated the phase transition from gas (G) state into the LC state at a lowest rate. This suggested a reduced packing effectiveness and fluidizing effect on DPPG molecules.

In the presence of green tea polyphenols, 2-hydroxy-5-methoxybenzaldehyde, apple E polyphenols and 2,4,6-trihydroxybenzaldehyde, the lift-off value increased to approximately 93 to 145 $Å^2$ per molecule and then the monolayer collapsed at about 45 to 52 $Å^2$ per molecule (collapse pressure around 40 to 55 mN/m), the slope of these isotherms was flatter compared to the isotherm obtained from pure water, which suggested the phase transition from gas (G) state into the LC state was smoother than that of the pure water. This again indicated a reduced packing effectiveness and fluidizing effect on DPPG molecules.

While the above compounds increased the lift off value and caused a flatter slope of the isotherms, 2,5-dihydroxybenzaldehyde was the only compound that decreased the lift off value, with a very small effect, to 88 $Å^2$ per molecule, which is considered not significant ($p > 0.05$), while the slope was still flatter compared to the isotherm obtained from water.

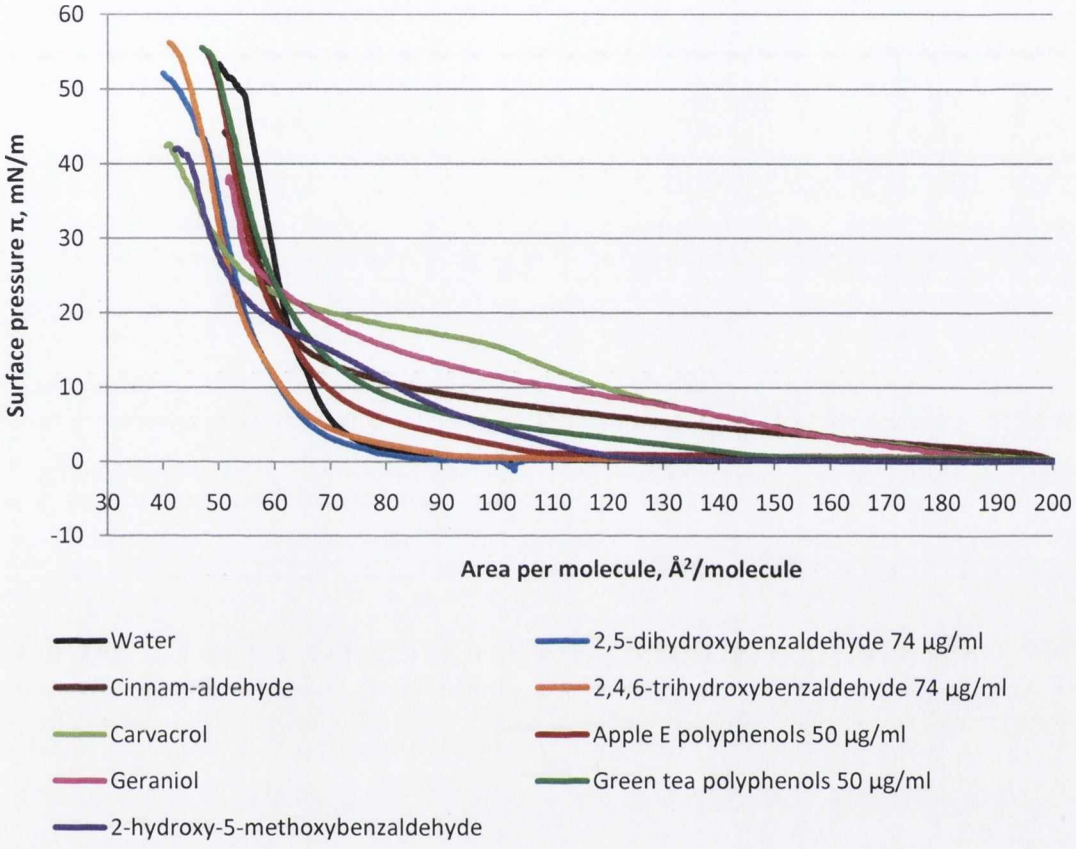

Figure 6.2.a The surface pressure-area (π-A) isotherms recorded for the monolayers formed by DPPG on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Analysis of the compression modulus for DPPG monolayer in the presence of selected naturally occurring antimicrobial compounds is shown in figure 6.2.b. For DPPG monolayer compressed on subphase containing pure water only, the maximal value of C_S^{-1} was observed at approximately 255 mN/m. All of the compounds tested caused a decrease in the maximal value of compression modulus compared to water, suggesting the compounds tested could fluidize the DPPG membrane, while carvacrol had the smallest compression modulus. That fluidizing effect caused by different compounds varied from ~100 to 195 mN/m. Among those compounds, carvacrol showed the greatest fluidizing effect. The next one was geraniol, which is also an oil compound. The fluidizing effect caused by the other compounds, including the other two oil compounds cinnamaldehyde and 2-hydroxy-5-methoxybenzaldehyde were found to be similar.

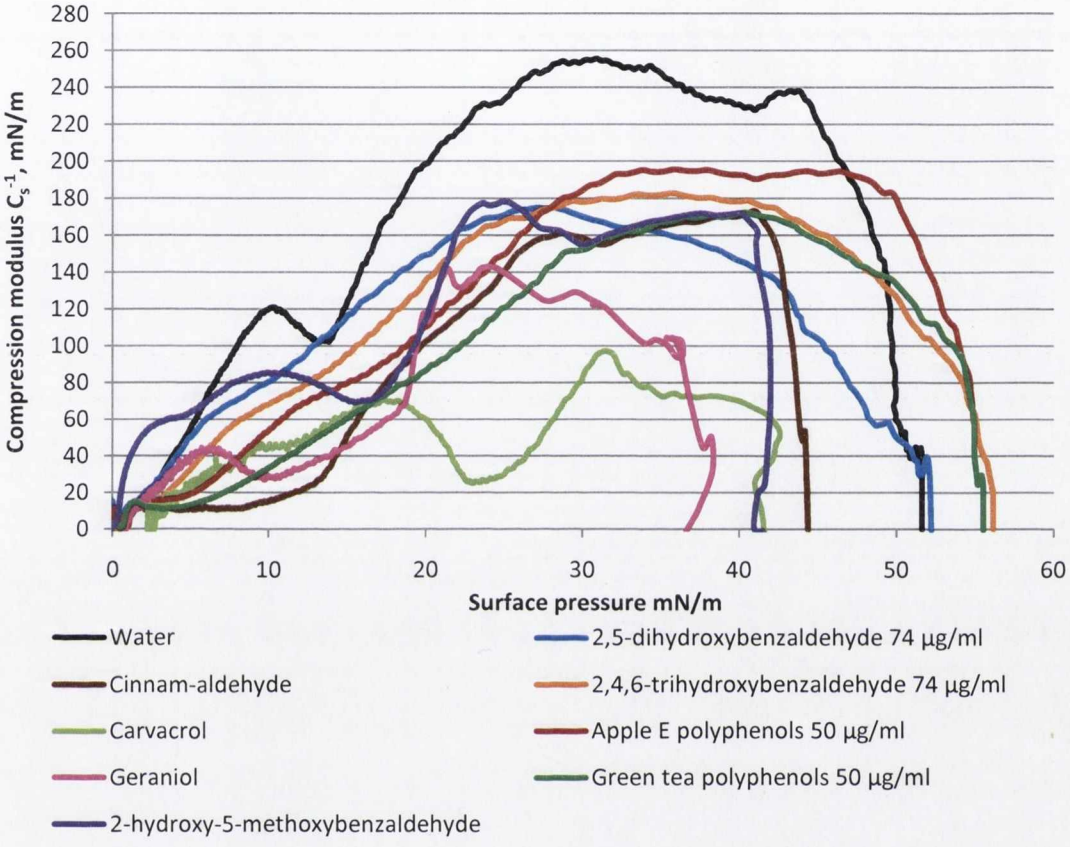

Figure 6.2.b The compression modulus (C_S^{-1}) values versus surface pressure (π) recorded for the monolayers formed by DPPG on subphases containing pure water with and without naturally occurring antimicrobial compounds.

The compression isotherms for cardiolipin monolayer deposited on subphases containing selected naturally occurring antimicrobial compounds are shown (figure 6.3.a). In subphase containing pure water only, the lift-off value was approximately 101 $Å^2$ per molecule and increased gradually into the liquid-condensed (LC) phase up to around 75 $Å^2$ per molecule, at which monolayer collapsed (collapse pressure was about 50 mN/m).

In contrast, naturally occurring antimicrobial compounds showed the lift-off value increased greatly to approximately 140 to 180 $Å^2$ per molecule. But the slope of these isotherms was flatter compared to the isotherm obtained from pure water, which indicated the phase transition from gas (G) state into the LC state was smoother than for pure water. This again suggested a reduced packing effectiveness and fluidizing effect on cardiolipin molecules. Among those compounds, carvacrol showed the highest lift-off value and the flattest slope, while 2,4,6-trihydroxybenzaldehyde showed the smallest increase of lift-off value and the steepest slope, despite this slope was the steepest among the compounds tested, it was still flatter compared to water.

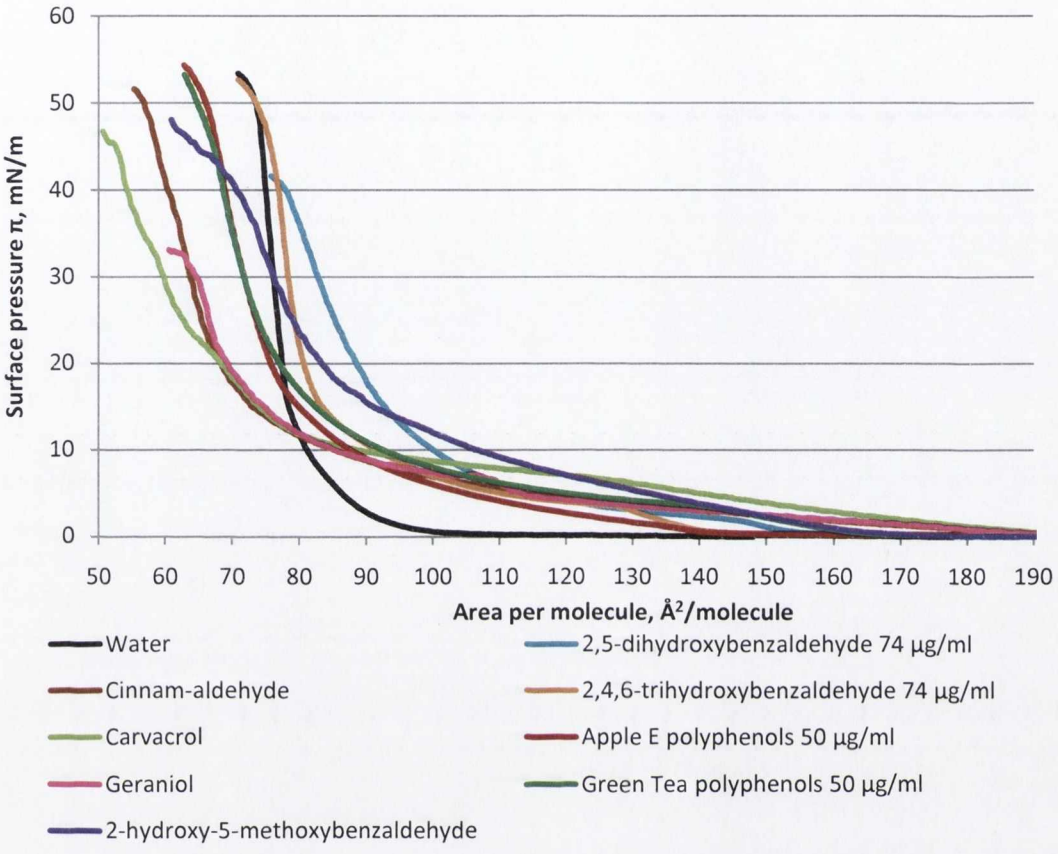

Figure 6.3.a The surface pressure-area (π-A) isotherms recorded for the monolayers formed by cardiolipin on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Analysis of the compression modulus for cardiolipin monolayer in the presence of selected naturally occurring antimicrobial compounds is shown in figure 6.3.b. For cardiolipin monolayer compressed on subphase containing pure water only, the maximal value of C_S^{-1} was observed at approximately 170 mN/m.

All of the compounds tested caused a decrease in the maximal value of compression modulus compared to water, suggesting the compounds tested could fluidized the cardiolipin membrane, and geraniol had the smallest compression modulus. The fluidizing effect caused by different compounds varied from ~75 to 150 mN/m. Among those compounds, geraniol showed the greatest fluidizing effect. The next was carvacrol, which is also an oil compound. The fluidizing effect caused by the powdered compounds, 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde, apple E polyphenols and green tea polyphenols were very similar.

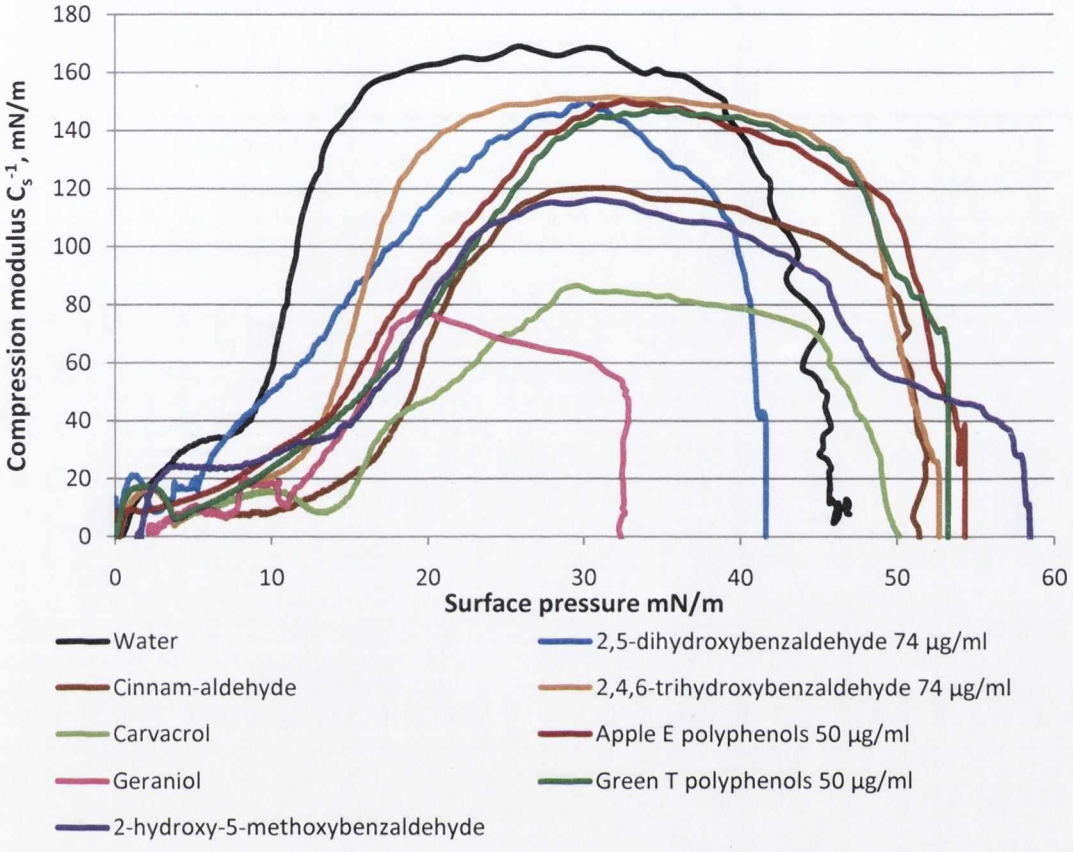

Figure 6.3.b The compression modulus (C_S^{-1}) values versus surface pressure (π) recorded for the monolayers formed by cardiolipin on subphases containing pure water with and without naturally occurring antimicrobial compounds.

The compression isotherms for DOPE monolayer deposited on subphases containing selected naturally occurring antimicrobial compounds are shown (figure 6.4.a). In subphase containing pure water only, the lift-off value was approximately 105 $Å^2$ per molecule and increased gradually into the liquid-condensed (LC) phase up to around 62 $Å^2$ per molecule, at which monolayer collapsed (collapse pressure was about 38 mN/m).

In contrast, isotherms of DOPE deposited on subphase with naturally occurring antimicrobial compounds showed the lift-off value increased and varied from approximately 110 to 200 $Å^2$ per molecule. In the presence of three oil compounds, cinnamaldehyde, carvacrol and geraniol, the slopes of the isotherms were the smallest.

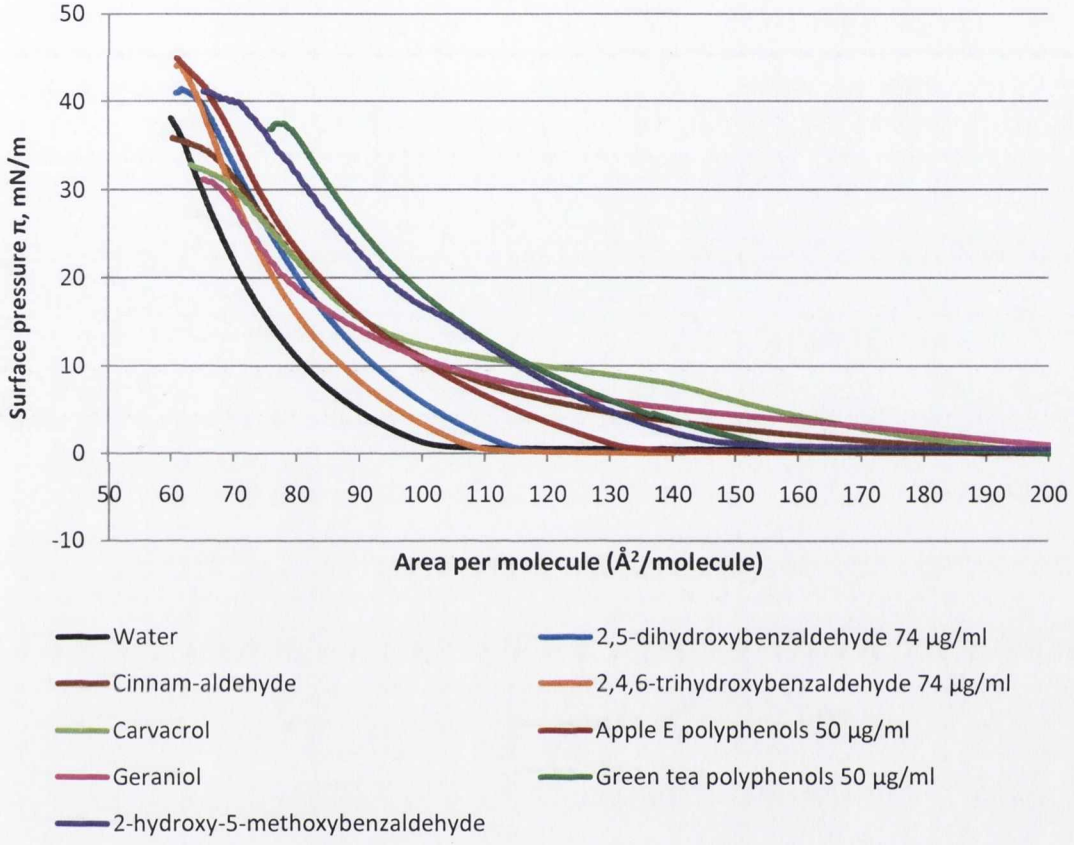

Figure 6.4.a The surface pressure-area (π-A) isotherms recorded for the monolayers formed by DOPE on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Analysis of the compression modulus for DOPE monolayer in the presence of different naturally occurring antimicrobial compounds is shown in figure 6.4.b. For DOPE monolayer compressed on subphase containing pure water only, the maximal value of C_S^{-1} was observed at approximately 122 mN/m, while 2,4,6-trihydroxybenzaldehyde was approximately 138 mN/m, suggesting a rigidifying effect of this compound on DOPE monolayer. For the remaining compounds, the maximal values of the compression modulus varied from approximately 50 to 110 mN/m. Such a decrease in the maximal value of the compression modulus indicated a fluidizing effect possibly caused by those naturally occurring antimicrobial compounds in the DOPE monolayer. Carvacrol showed the highest fluidizing effect, while apple E polyphenols and 2,5-dihydroxybenzaldehyde exhibited the least fluidizing effect.

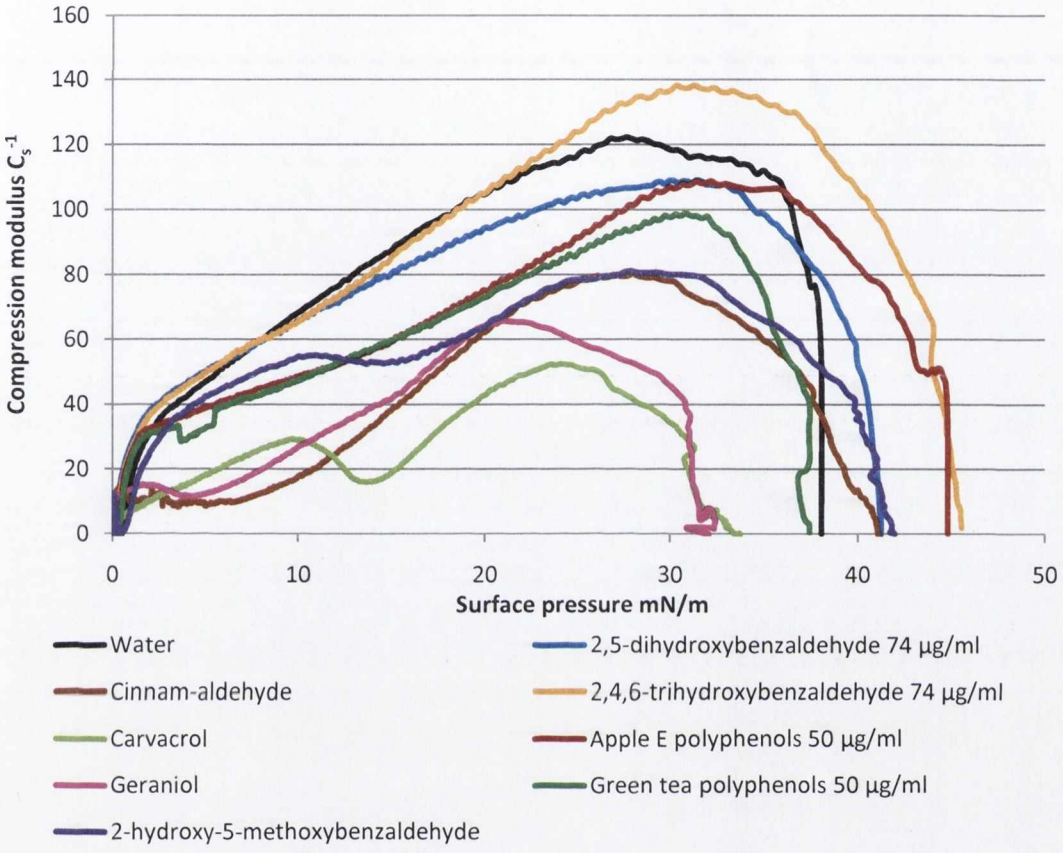

Figure 6.4.b The compression modulus (C_S^{-1}) values versus surface pressure (π) recorded for the monolayers formed by DOPE on subphases containing pure water with and without naturally occurring antimicrobial compounds.

The compression isotherms for DOPG monolayer deposited on subphases containing selected naturally occurring antimicrobial compounds are shown (figure 6.5.a). In subphase containing pure water only, the lift-off value was approximately 120 $Å^2$ per molecule and increased gradually into the liquid-condensed (LC) phase up to around 68 $Å^2$ per molecule, at which monolayer collapsed (collapse pressure was about 40 mN/m).

In contrast, isotherms of DOPG deposited on subphase with naturally occurring antimicrobial compounds showed the lift-off value varied from approximately 117 to 195 $Å^2$ per molecule. In the presence of two of the oil compounds, carvacrol and geraniol, the slope of the isotherms were the smallest which indicated a reduced packing effectiveness and fluidizing effect on DOPG molecules. Powdered compounds, 2,4,6-trihydroxybenzaldehyde and 2,5-dihydroxybenzaldehyde caused a slight decrease in the lift-off value, however, such decrease was not significant ($p > 0.05$).

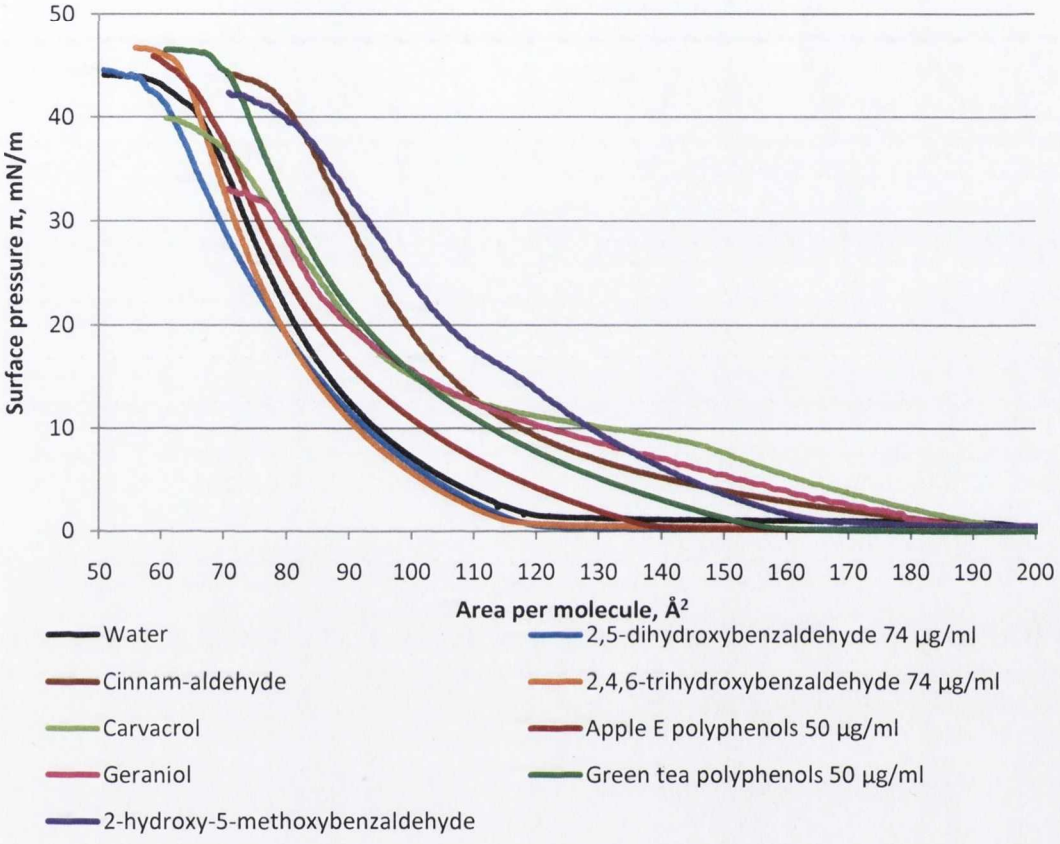

Figure 6.5.a The surface pressure-area (π-A) isotherms recorded for the monolayers formed by DOPG on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Analysis of the compression modulus for DOPG monolayer in the presence of different naturally occurring antimicrobial compounds is shown in figure 6.5.b. For DOPG monolayer compressed on subphase containing pure water only, the maximal value of C_S^{-1} was observed at approximately 118 mN/m, while 2,4,6-trihydroxybenzaldehyde was approximately 137 mN/m, indicating a rigidifying effect. For the remaining compounds, the maximal values of the compression modulus varied from approximately 42 to 117 mN/m. Such a decrease in the maximal value of the compression modulus suggested a fluidizing effect caused by those naturally occurring antimicrobial compounds in the DOPG monolayer. Geraniol showed the highest fluidizing effect, while apple E polyphenols showed the least fluidizing effect and green tea polyphenols showed almost the same maximal value as water.

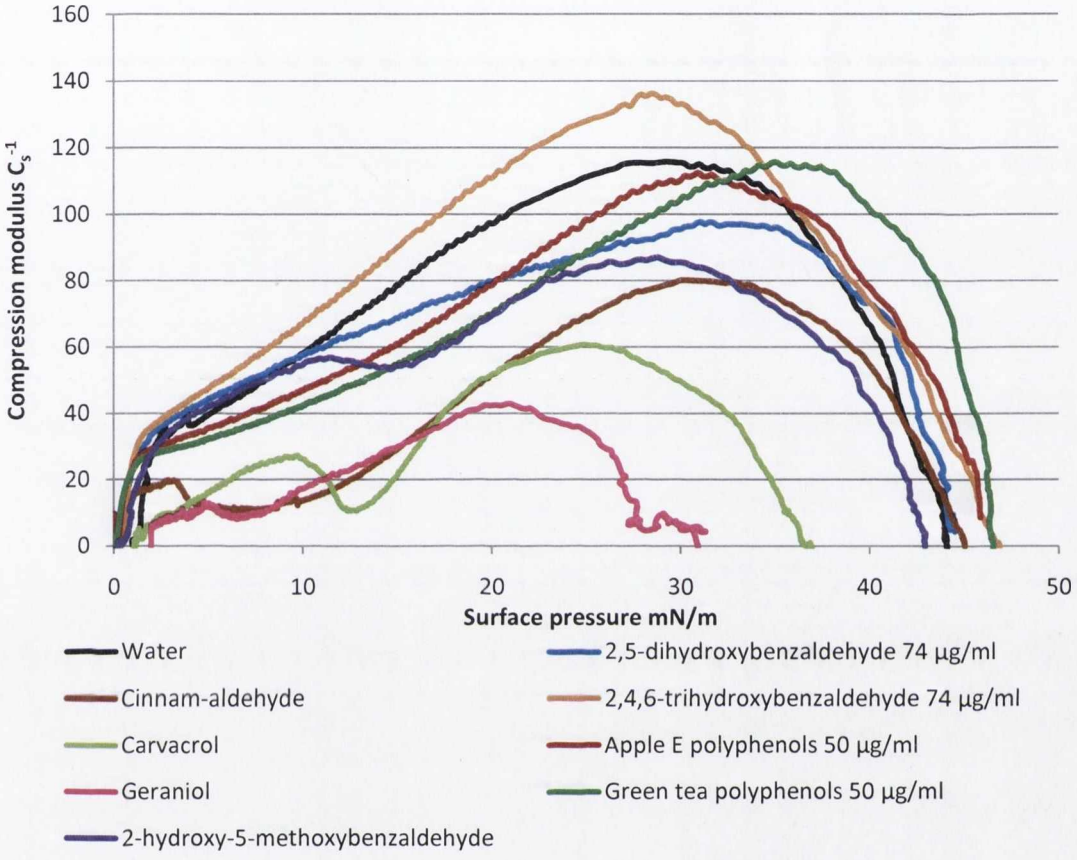

Figure 6.5.b The compression modulus (C_S^{-1}) values versus surface pressure (π) recorded for the monolayers formed by DOPG on subphases containing pure water with and without naturally occurring antimicrobial compounds.

6.3.2 Surface potential

Figure 6.6 shows the changes of surface potential of DPPE monolayer on subphase containing pure water with and without natural antimicrobial compounds. At the gas phase, lipids were lying randomly (the orientation of the lipid could range from between almost 0° (parallel) to 90° (perpendicular)) on the surface of the subphase. During compression, phase transition of lipids occurred, which changed from gas state to liquid state, thus increased the dipole density per unit area and caused an increase of surface potential. The value of surface potential increased from the dipole moment, which suggested the formation of domains by zwitterionic phospholipid DPPE molecules.

Considering the monolayer of DPPE at condensed state (pressure = ~30 mN/m and area per molecue = ~30 - 40 $Å^2$/molecule), it was found that the surface potential of DPPE monolayer on water reached up to approximately 600 mV. In the presence of 2-hydroxy-5-methoxybenzaldehyde, the surface potential of DPPE reached up to ~600 mV despite the starting point of increase changed from 125 $Å^2$/molecule to 75 $Å^2$/molecule. In the presence of carvacrol, the surface potential of DPPE could only increase up to ~500 mV and in the presence of 2,5-dihydroxybenzaldehyde, apple E polyphenols, green tea polyphenols, cinnamaldehyde and geraniol, the surface potential of DPPE was however reduced by 50%, which was ~300 mV. This decrease suggests there was a change in the orientation and interaction of the antimicrobial-lipid complexes on the lipid molecular packing, which led to a decrease in dipole density of the DPPE monolayer. The lower the surface potential, the lower the value of the total dipole moment.

Cinnamaldehyde showed strong antimicrobial effect on Gram-positive organism, *Map* (BA_{50} 24 μg/ml) and moderate effect on Gram-negative organism *E. coli* (BA_{50} 112 μg/ml). Geraniol showed the least antimicrobial effects on both Gram-positive organism, *Cl. sporogenes* (341 μg/ml) and Gram-negative organism, *E. coli* (450 μg/ml), however, it was capable to lower the surface potential of DPPE monolayer to a similar extend as cinnamaldehyde. It might due to the natural biological membrane in living bacteria is much more complex than the single component DPPE monolayer, despite DPPE is the dominated phospholipid in *E. coli*.

Though the values of the final reading of surface potential measurement were similar in the presence of cinnamaldehyde and geraniol, the way of the reorganization of the lipid molecules and their rafts structure might be different, as the dipole moment of the model membrane system had arisen from three components, including the contribution from the reorientation of water molecules in the aqueous phase, the contribution from the head groups, and the contribution from the hydrophobic tails of the lipid molecules.

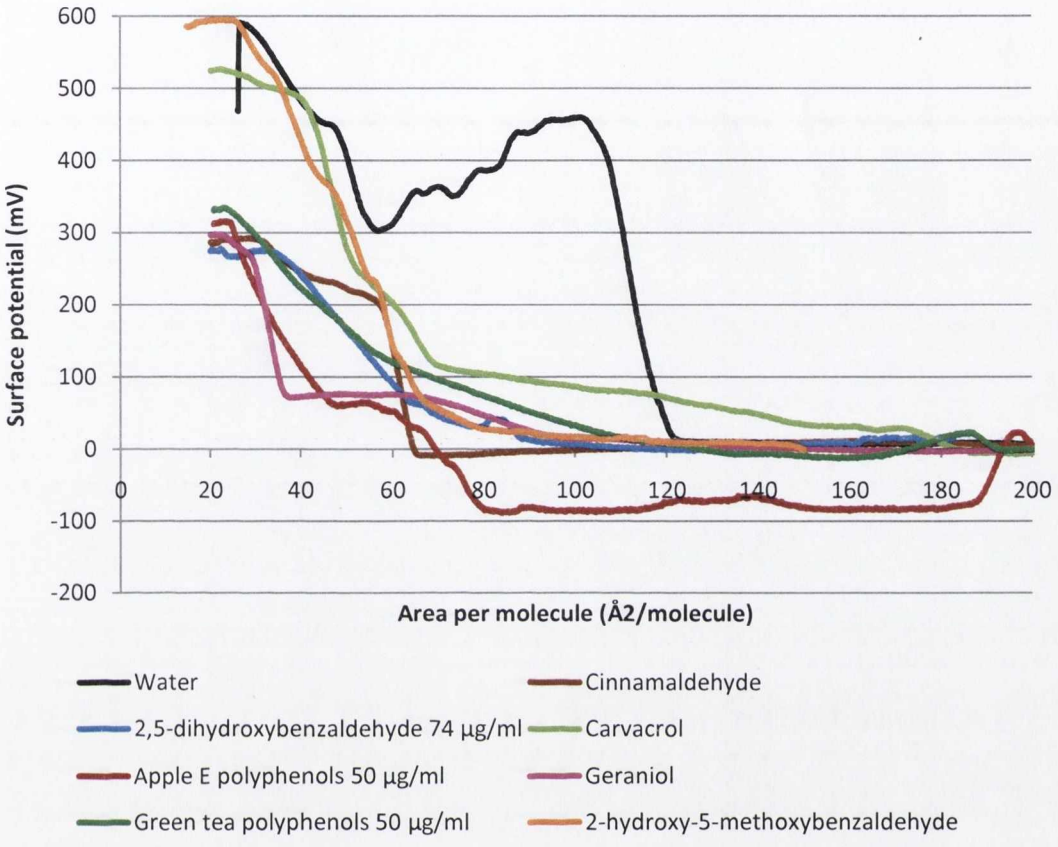

Figure 6.6 The surface potential measurement versus molecular area recorded for the monolayers formed by DPPE on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Figure 6.7 shows the changes of surface potential of DPPG monolayer on subphase containing pure water with and without natural antimicrobial compounds. Similar to the DPPE monolayer, the value of surface potential raised from the dipole moment during compression, which indicated the formation of domains by anionic phospholipid DPPG molecules.

Considering the monolayer of DPPG at condensed state (pressure = ~30 mN/m and area per molecue = ~40 - 80 $Å^2$/molecule), it was found that the surface potential of DPPG monolayer on water reached up to approximately ~450 mV. In the presence of natural antimicrobials, 2-hydroxy-5-methoxybenzaldehyde and apple E polyphenols, the final readings of surface potential of DPPG increased slightly but the starting points of increase changed from ~125 $Å^2$/molecule to ~250 and ~150 $Å^2$/molecule respectively.

In the presence of green tea polyphenols, the surface potential of DPPG dropped slightly to ~380 mV, 2,5-dihydroxybenzaldehyde and carvacrol resulted in the decrease of the surface potential of DPPG to ~230 and ~180 mV respectively and cinnamaldehyde and geraniol cause a drop in the surface potential of DPPG over 50%, to ~75 and ~25 mV respectively, suggesting a decrease in dipole moment of the DPPG monolayer and a change in the orientation and interaction of the antimicrobial-lipid complexes on the lipid molecular packing.

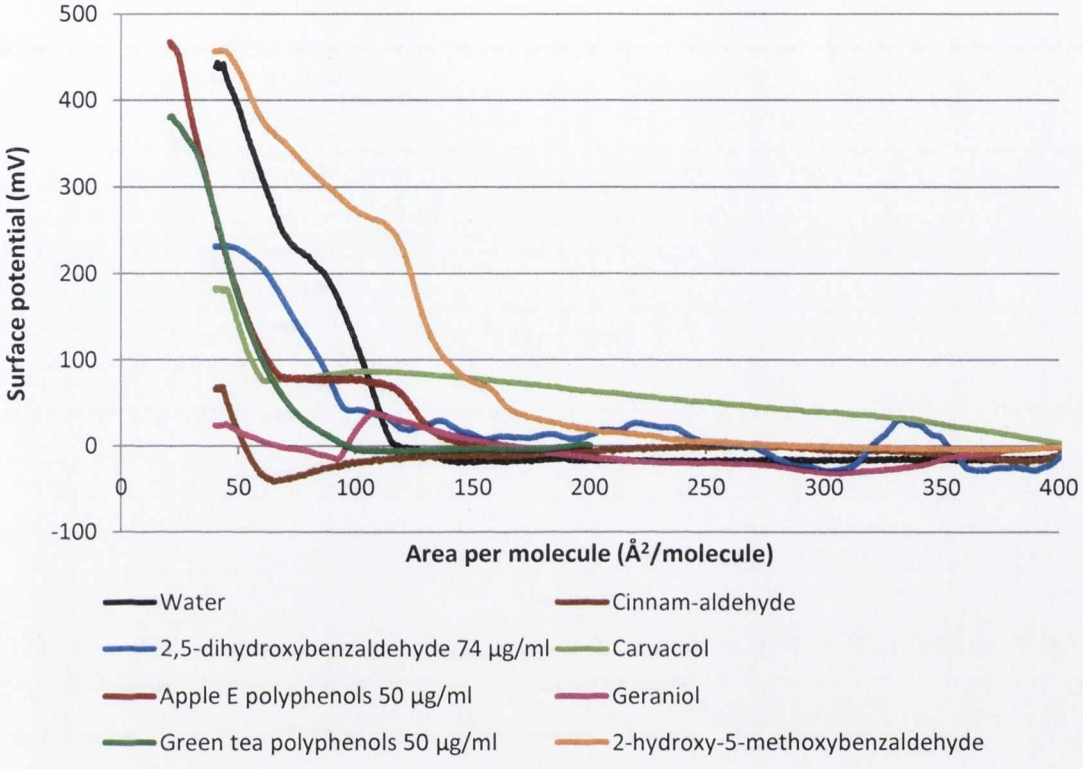

Figure 6.7 The surface potential measurement versus molecular area recorded for the monolayers formed by DPPG on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Figure 6.8 shows the changes of surface potential of cardiolipin monolayer on subphase containing pure water with and without natural antimicrobial compounds. Similar to the other monolayers, the value of surface potential increased from the dipole moment during compression, which indicated the formation of domains by anionic phospholipid cardiolipin molecules.

Considering the monolayer of cardiolipin at condensed state (pressure = ~30 mN/m and area per molecue = ~50 – 100 $Å^2$/molecule), it was found that the surface potential of cardiolipin monolayer on water could reach up to approximately 250 mV. On the other hand, natural antimicrobials 2-hydroxy-5-methoxybenzaldehyde, 2,5-dihydroxybenzaldehyde, apple E polyphenols and green tea polyphenols could greatly increase the surface potential of cardiolipin monolayer to approximately 380 – 450 mV, while carvacrol increased the surface potential slightly to 280 mV.

Cinnamaldehyde and geraniol could lower the surface potential and geraniol produced a negative final reading. As mentioned in the result of DOPG monolayer, a decrease in surface potential suggests a decrease in dipole moment of the cardiolipin monolayer, while a negative value of surface potential will suggest a change in the orientation of the dipoles, i.e. reversing the orientation of dipole moments of molecules (antimicrobial-lipid aggregates) in the monolayer, the higher the magnitude, the higher the dipole moment, regardless if the value has a positive or negative sign.

This decrease therefore suggested there were changes in the orientation of lipid molecules, adjacent water molecules, charge distribution and the negative surface potential reading might also indicated that the orientation of the dipoles had rotated by 180°.

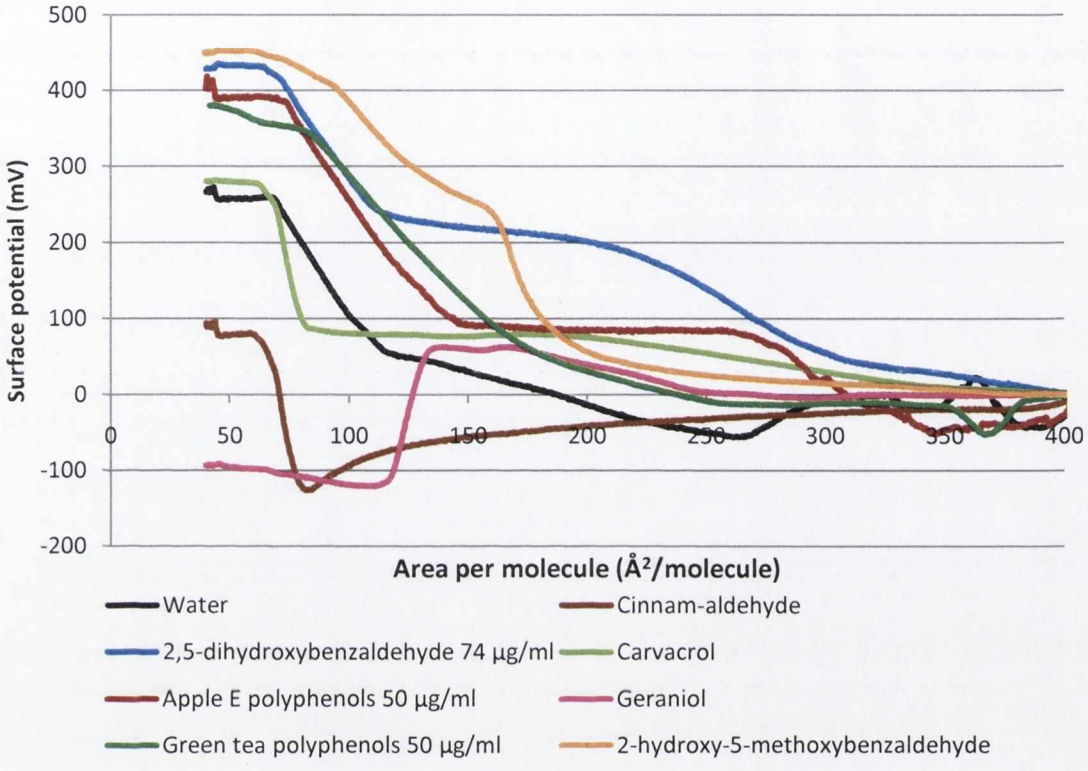

Figure 6.8 The surface potential measurement versus molecular area recorded for the monolayers formed by cardiolipin on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Figure 6.9 shows the changes of surface potential of DOPE monolayer on subphase containing pure water with and without natural antimicrobial compounds. Same as to the DPPE monolayer, before compression, DOPE lipids were lying randomly on the surface of the subphase. As phase transition of lipids occurred, which changed from gas state to liquid state, the dipole density increased and so as the surface potential. The value of surface potential rose from the dipole moment, which reflected the formation of domains by zwitterionic phospholipid DOPE molecules.

Considering the monolayer of DOPE at condensed state (pressure = ~30 mN/m and area per molecule = ~50 - 80 $Å^2$/molecule), it was observed that the surface potential of DOPE monolayer on water reached up to approximately 175 mV. In the presence of natural antimicrobial, 2-hydroxy-5-methoxybenzaldehyde and carvacrol, the final readings of surface potential of DOPE increased to ~375 mV and ~275 mV respectively and the starting points of increase changed from ~ 125 $Å^2$/molecule to ~250 $Å^2$/molecule, i.e. the surface potential started to lift off much earlier than DOPE on pure water or with other natural antimicrobials. This early lift off reading indicated that 2-hydroxy-5-methoxybenzaldehyde and carvarol started to interact with the DOPE molecules at the early stage when the DOPE molecules was still in gas state and thus the dipole moment in the membrane system was therefore induced earlier.

In the presence of apple E polyphenols and green tea polyphenols, the surface potential of DOPE also increased to ~200 mV and ~210 mV respectively, but comparing to 2-hydroxy-5-methoxybenzaldehyde and carvacrol, the increase was much smaller and not significant ($p > 0.05$). Such increase in surface potential suggested an increase in dipole moment of the DOPE monolayer. In the presence of 2,5-dihydroxybenzaldehyde, the surface potential of DOPE decreased slightly to ~160 mV, also considered not significant ($p > 0.05$); and in the presence of cinnamaldehyde and geraniol, the surface potential of DOPE decreased over 50%, which was ~75 mV, suggesting a decrease in dipole moment of the DOPE monolayer.

For DOPE monolayer, 2-hydroxy-5-methoxybenzaldehyde and carvacrol might have incorporated into the liquid-expanded phase, interacting with the lipid molecules and modify the molecular packing and organization of the lipid domains. While the decrease caused by cinnamaldehyde and geraniol suggested these antimicrobials might have incorporated into the monolayer at the liquid-condensed phase.

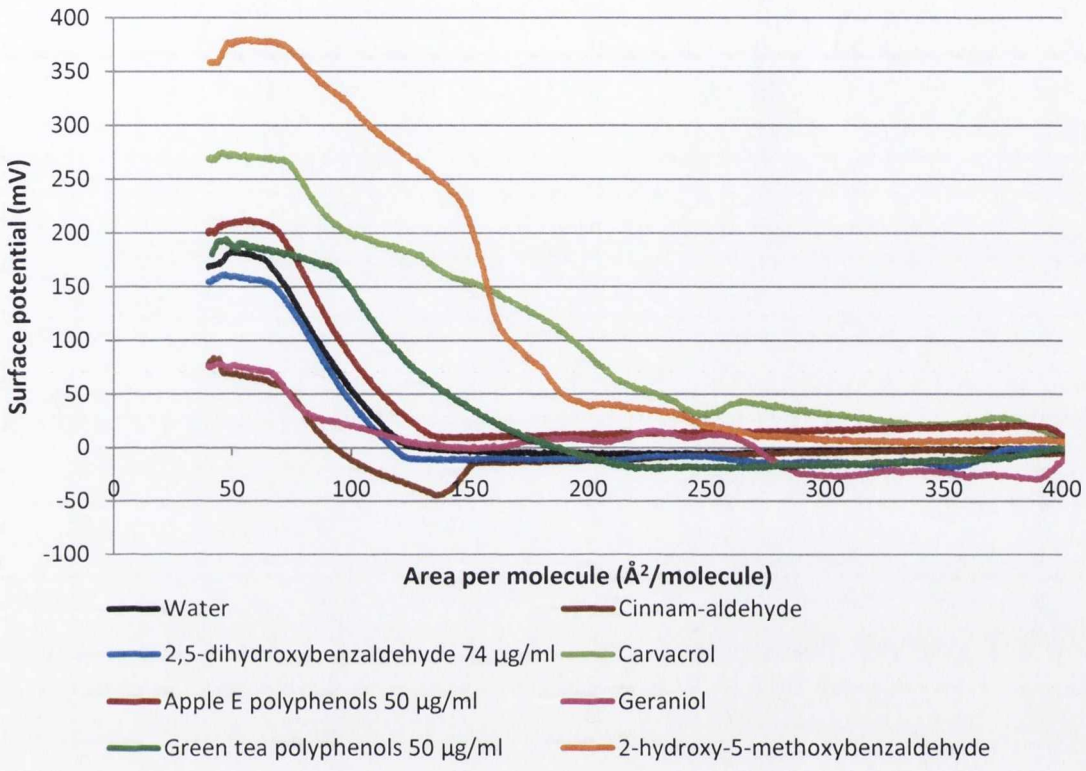

Figure 6.9 The surface potential measurement versus molecular area recorded for the monolayers formed by DOPE on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Figure 6.10 shows the changes of surface potential of DOPG monolayer on subphase containing pure water with and without natural antimicrobial compounds. The value of surface potential raised from the dipole moment during compression which indicated the formation of domains by anionic phospholipid DOPG molecules.

Considering the monolayer of DOPG at condensed state (pressure = ~30 mN/m and area per molecue = ~50 - 100 $Å^2$/molecule), it was found that the surface potential of DOPG monolayer on water could reached up to approximately 160 mV. In the presence of natural antimicrobials, 2-hydroxy-5-methoxybenzaldehyde, the final reading of surface potential of DOPG increased greatly to approximately 275 mV. In the presence of apple E polyphenols and green tea polyphenols, the surface potential also increased to ~190 mV and ~200 mV respectively, but comparing to 2-hydroxy-5-methoxybenzaldehyde, the effect of increase was much smaller and the effect caused by apple E polyphenols was considered not significant ($p > 0.05$). Such increase in surface potential suggested an increase in dipole moment of the DOPG monolayer.

In the presence of 2,5-dihydroxybenzaldehyde, the surface potential of DOPG reached up to ~160 mV. In the presence of carvacrol, cinnamaldehyde and geraniol, a decrease in the surface potential of DOPG was observed, over 50% for carvacrol, approximately 50 mV, while for cinnamaldehyde and geraniol, the surface potential of DOPG was approximately –65 and –200 mV respectively. A decrease on surface potential caused by carvacrol suggested a decrease in dipole moment of the DOPG monolayer, while a negative value of surface potential caused by cinnamaldehyde and geraniol suggested a change in the orientation of the lipid molecules, i.e. reversing the orientation of dipole moments of (antimicrobial-lipid aggregates) in the monolayer, the higher the magnitude, the higher the dipole moment, regardless if the value has a positive or negative sign.

These positive and negative readings suggested the changes in the orientation of lipid molecules, adjacent water molecules and charge distribution were large and the negative surface potential reading implies that the orientation of the dipoles might have rotated by 180°.

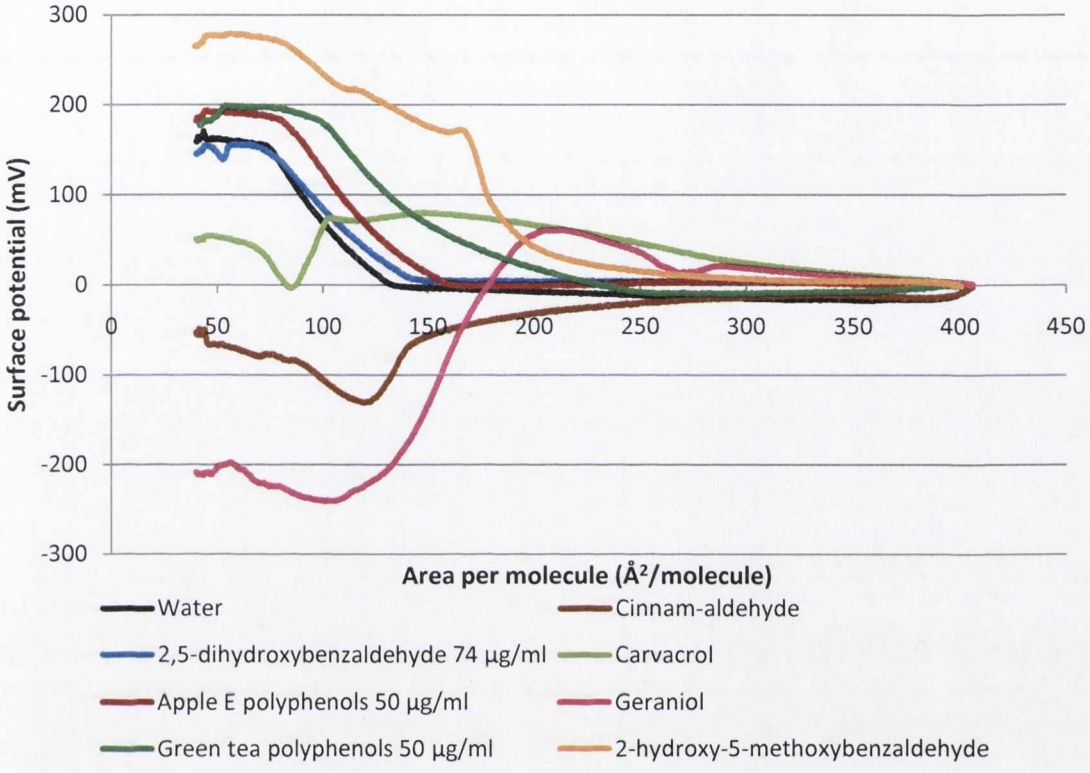

Figure 6.10 The surface potential measurement versus molecular area recorded for the monolayers formed by DOPG on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Figure 6.11 shows the changes of surface potential of monolayer composed of total lipids extracted from *Map* culture on subphase containing pure water with and without natural antimicrobial compounds. Similar to the findings observed from the other monolayers, the value of surface potential raised from the dipole moment during compression, which reflected the formation of domains by anionic phospholipid cardiolipin molecules.

Considering the surface potential measurement at condensed state (pressure = ~30 mN/m), it was noted that the surface potential of the monolayer of total lipids extracted from *Map* cultures on water reached up to approximately 360 mV. In the presence of anti-*Map* natural compounds, 2-hydroxy-5-methoxybenzaldehyde, the trend of the surface potential measurement remained similar. In the presence of other anti-*Map* natural compounds, 2,5-dihydroxybenzaldehyde, carvacrol, cinnamaldehyde and negative control, geraniol, the surface potential decreased and negative readings were observed at the liquid-condensed state in the presence of cinnamaldehyde and geraniol. This decrease suggested there was a change in the orientation and interaction of the antimicrobial-lipid complexes on the lipid molecular packing.

2,5-dihydroxybenzaldehyde, carvacrol, cinnamaldehyde and geraniol all lowered the final readings of the surface potential and geraniol produced a negative final reading. As mentioned previously, a decrease in surface potential suggests a decrease in dipole moment of the monolayer, while a negative value of surface potential suggests a change in the orientation of the lipid molecules, i.e. reversing the orientation of dipole moments of (antimicrobial-lipid aggregates) in the monolayer, the higher the magnitude, the higher the dipole moment, regardless if the value has a positive or negative sign.

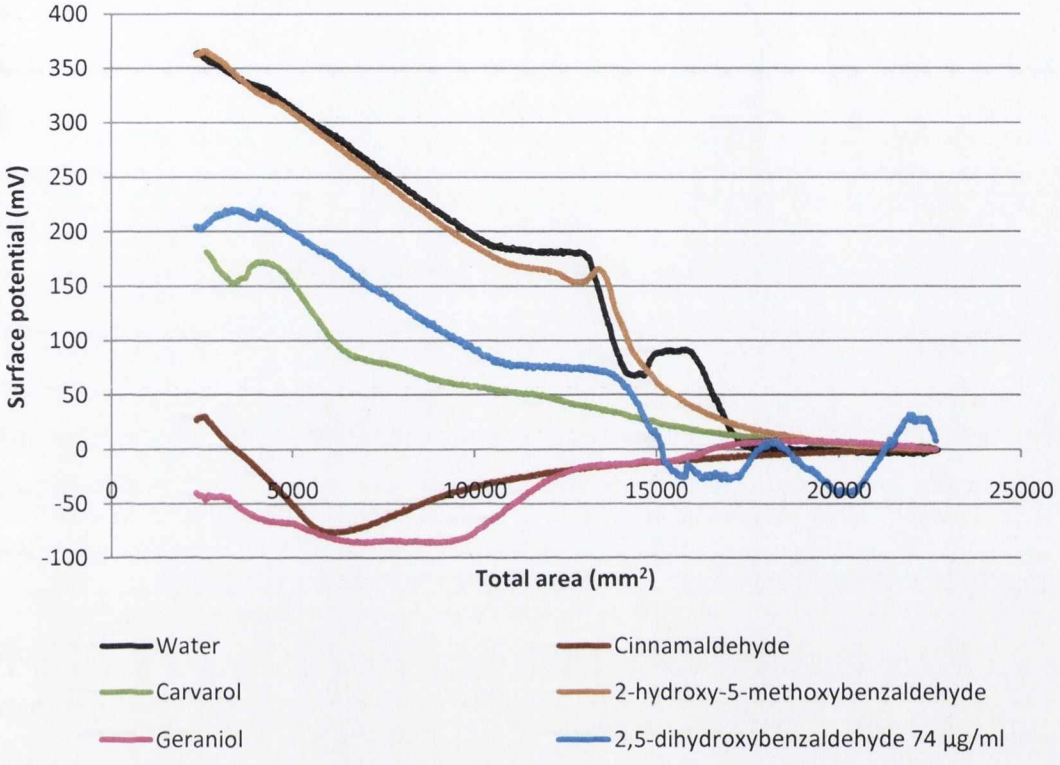

Figure 6.11 The surface potential measurement versus total area recorded for the monolayers formed by total lipids extracted from *Map* culture on subphases containing pure water with and without naturally occurring antimicrobial compounds.

Tables 6.1 to 6.5 show the lift off values, values of collapse area and pressure, maximal values of compression modulus and readings of surface potential at the condensed state of different monolayers compressed on subphase containing pure water with and without naturally occurring antibacterial compound. Statistical analysis, ordinary ANOVA was performed by using GraphPad InStat (GraphPad Software, Inc., La Jolla, CA, USA) to compare whether these values represent significant effect caused by the presence of naturally occurring antibacterial compound in the subphase.

Table 6.1 shows the recorded lift off values of different monolayers, on subphase containing pure water with and without naturally occurring antibacterial compound. The lift off value indicates the beginning of observable interactions between lipid molecules, i.e. the phase transition from gas state to liquid expanded state. The larger the value, the earlier the lipid molecules start to interact with each other.

For DPPE monolayer, compounds green tea polyphenols, *trans*-cinnalaldehyde, carvarol and geraniol significantly increased ($p < 0.001$) the lift off values, which indicated that these compounds caused the lipid molecules to interact earlier during the compression. Comparing the effect of the above four compounds, the level of increase of lift off value caused by *trans*-cinnalaldehyde, carvarol and geraniol was similar, there is no significant difference ($p > 0.05$) between them, however, the level of increase of lift off value caused by green tea polyphenols was significantly different ($p < 0.001$) among the four compounds.

On the other hand, compounds 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde and apple E polyphenols significantly decreased the lift off value ($p < 0.001$), which indicated that these compounds caused a delay in phase transition. The effects of 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde and apple E polyphenols were not significantly different ($p < 0.001$). Compound 2-hydroxy-5-methoxybenzaldehyde did not cause significant effect on the lift off value of DPPE monolayer ($p > 0.05$).

For DOPE monolayer, compounds 2,5-dihydroxybenzaldehyde, apple E polyphenols, green tea polyphenols, *trans*-cinnalaldehyde, carvarol, geraniol and 2-hydroxy-5-methoxybenzaldehyde significantly increased the lift off value ($p < 0.05$) comparing to subphase containing pure water only, while 2,4,6-trihydroxybenzaldehyde did not cause significant effect on the lift off value of DOPE monolayer ($p > 0.05$). The compounds causing the most significant increase of the lift off value were in the order of *trans*-cinnalaldehyde, carvarol and geraniol > green tea polyphenols and 2-hydroxy-5-methoxybenzaldehyde > apple E polyphenols > 2,5-dihydroxybenzaldehyde.

For DPPG monolayer, compounds apple E polyphenols, green tea polyphenols, *trans*-cinnalaldehyde, carvarol, geraniol and 2-hydroxy-5-methoxybenzaldehyde significantly increased the lift off value ($p < 0.01$) comparing to subphase containing pure water only, while 2,5-dihydroxybenzaldehyde and 2,4,6-trihydroxybenzaldehyde did not cause significant effect on the lift off value of DPPG monolayer ($p > 0.05$). The compounds causing the most significant increase of the lift off value were in the order of *trans*-cinnalaldehyde and carvarol > geraniol > green tea polyphenols > 2-hydroxy-5-methoxybenzaldehyde > apple E polyphenols.

For DOPG monolayer, compounds apple E polyphenols, green tea polyphenols, *trans*-cinnalaldehyde, carvarol, geraniol and 2-hydroxy-5-methoxybenzaldehyde significantly increased the lift off value ($p < 0.001$) comparing to subphase containing pure water only, while 2,5-dihydroxybenzaldehyde and 2,4,6-trihydroxybenzaldehyde did not cause significant effect on the lift off value of DOPG monolayer ($p > 0.05$). These effects are similar to those observed in DPPG monolayer. The compounds causing the most significant increase of the lift off value were in the order of *trans*-cinnalaldehyde, carvarol and geraniol > 2-hydroxy-5-methoxybenzaldehyde > green tea polyphenols > apple E polyphenols.

For CL monolayer, all the compounds 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, apple E polyphenols, green tea polyphenols, *trans*-cinnalaldehyde, carvarol, geraniol and 2-hydroxy-5-methoxybenzaldehyde significantly

increased the lift off value ($p < 0.001$ to 0.01) comparing to subphase containing pure water only. Their effects were in the order of *trans*-cinnalaldehyde, carvarol and geraniol > 2-hydroxy-5-methoxybenzaldehyde, green tea polyphenols, apple E polyphenols, 2,5-dihydroxybenzaldehyde and 2,4,6-trihydroxybenzaldehyde.

Table 6.1 The lift off values ($Å^2$) of lipid monolayers DPPE, DOPE, DPPG, DOPG and CL compressed on subphases containing pure water with and without naturally occurring antimicrobial compounds.

	DPPE	DOPE	DPPG	DOPG	CL
Water	**47**	**105**	**88**	**120**	**101**
2,5-Dihydroxy benzaldehyde	33 Significantly decreased ($p < 0.001$)	116 Significantly increased ($p < 0.05$)	87 No significant effect ($p > 0.05$)	120 No significant effect ($p > 0.05$)	154 Significantly increased ($p < 0.001$)
2,4,6-Trihydroxy benzaldehyde	29 Significantly decreased ($p < 0.001$)	110 No significant effect ($p > 0.05$)	95 No significant effect ($p > 0.05$)	117 No significant effect ($p > 0.05$)	142 Significantly increased ($p < 0.001$)
Apple E polyphenols	35 Significantly decreased ($p < 0.001$)	134 Significantly increased ($p < 0.001$)	110 Significantly increased ($p < 0.01$)	139 Significantly increased ($p < 0.001$)	141 Significantly increased ($p < 0.01$)
Green tea polyphenols	64 Significantly increased ($p < 0.001$)	157 Significantly increased ($p < 0.001$)	148 Significantly increased ($p < 0.001$)	158 Significantly increased ($p < 0.001$)	153 Significantly increased ($p < 0.001$)
***trans*-Cinnamaldehyde**	117 Significantly increased ($p < 0.001$)	185 Significantly increased ($p < 0.001$)	200 Significantly increased ($p < 0.001$)	189 Significantly increased ($p < 0.001$)	180 Significantly increased ($p < 0.001$)
Carvacrol	115 Significantly increased ($p < 0.001$)	190 Significantly increased ($p < 0.001$)	195 Significantly increased ($p < 0.001$)	194 Significantly increased ($p < 0.001$)	190 Significantly increased ($p < 0.001$)
Geraniol	115 Significantly increased ($p < 0.001$)	200 Significantly increased ($p < 0.001$)	179 Significantly increased ($p < 0.001$)	189 Significantly increased ($p < 0.001$)	190 Significantly increased ($p < 0.001$)
2-Hydroxy-5-methoxy benzaldehyde	47 No significant effect ($p > 0.05$)	155 Significantly increased ($p < 0.001$)	124 Significantly increased ($p < 0.001$)	165 Significantly increased ($p < 0.001$)	164 Significantly increased ($p < 0.001$)

Table 6.2 shows the recorded values of collapse area of different monolayers, on subphase containing pure water with and without naturally occurring antibacterial compound. The value of collapse area represents the smallest area occupied by each lipid molecule, which can be compressed at condensed state, just before the formation of lipid multi-layers, i.e. the collapse of monolayer (as illustrated in figure 6.13). A smaller collapse area indicates the lipid molecules could be compressed/packed closer to each other in the monolayer without collapsing.

For DPPE monolayer, among all compounds tested, only apple E polyphenols showed a significant decrease ($p < 0.05$) in the collapse area. For DOPE monolayer, only green tea polyphenols showed a significant increase ($p < 0.01$) in the collapse area. For DPPG monolayer, a significant decrease was observed with 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, carvarol ($p < 0.01$) and 2-hydroxy-5-methoxybenzaldehyde ($p < 0.05$ in the collapse area. These compounds had caused similar degrees of decrease and there was no significant difference ($p > 0.05$) between them. For DOPG monolayer, *trans*-cinnalaldehyde, geraniol and 2-hydroxy-5-methoxybenzaldehyde showed a significant increase ($p < 0.05$) in the collapse area with a similar level of effect ($p > 0.05$). For Cl monolayer, only *trans*-cinnalaldehyde ($p < 0.01$) and carvacrol ($p < 0.001$) showed a significant decrease in the collapse area. When comparing the two compounds, there was no significant difference ($p > 0.05$) between the effect they caused.

Table 6.2 The values of collapse area ($Å^2$) of lipid monolayers DPPE, DOPE, DPPG, DOPG and CL compressed on subphases containing pure water with and without naturally occurring antimicrobial compounds.

	DPPE	DOPE	DPPG	DOPG	CL
Water	**25**	**61**	**55**	**68**	**74**
2,5-Dihydroxy benzaldehyde	19 No significant effect ($p > 0.05$)	65 No significant effect ($p > 0.05$)	42 Significantly decreased ($p < 0.01$)	61 No significant effect ($p > 0.05$)	78 No significant effect ($p > 0.05$)
2,4,6-Trihydroxy benzaldehyde	19 No significant effect ($p > 0.05$)	63 No significant effect ($p > 0.05$)	43 Significantly decreased ($p < 0.01$)	62 No significant effect ($p > 0.05$)	75 No significant effect ($p > 0.05$)
Apple E polyphenols	14 Significantly decreased ($p < 0.05$)	66 No significant effect ($p > 0.05$)	49 No significant effect ($p > 0.05$)	66 No significant effect ($p > 0.05$)	66 No significant effect ($p > 0.05$)
Green tea polyphenols	23 No significant effect ($p > 0.05$)	79 Significantly increased ($p < 0.01$)	49 No significant effect ($p > 0.05$)	71 No significant effect ($p > 0.05$)	67 No significant effect ($p > 0.05$)
***trans*-Cinnamaldehyde**	29 No significant effect ($p > 0.05$)	66 No significant effect ($p > 0.05$)	53 No significant effect ($p > 0.05$)	79 Significantly increased ($p < 0.05$)	57 Significantly decreased ($p < 0.01$)
Carvacrol	28 No significant effect ($p > 0.05$)	68 No significant effect ($p > 0.05$)	42 Significantly decreased ($p < 0.01$)	71 No significant effect ($p > 0.05$)	53 Significantly decreased ($p < 0.001$)
Geraniol	27 No significant effect ($p > 0.05$)	67 No significant effect ($p > 0.05$)	52 No significant effect ($p > 0.05$)	78 Significantly increased ($p < 0.05$)	64 No significant effect ($p > 0.05$)
2-Hydroxy-5-methoxy benzaldehyde	25 No significant effect ($p > 0.05$)	71 No significant effect ($p > 0.05$)	45 Significantly decreased ($p < 0.05$)	81 Significantly increased ($p < 0.05$)	70 No significant effect ($p > 0.05$)

Table 6.3 shows the recorded values of collapse pressure of different monolayers, on subphase containing pure water with and without naturally occurring antibacterial compound. The collapse pressure is the highest pressure which can be achieved by the lipid monolayer at condensed state just before the breakage of monolayer structure. The collapse pressure is one of the factor that determines the morphology and properties of the monolayer. A higher collapse pressure indicates a stronger attraction between the neighbor molecules at the condensed state.

For DPPE monolayer, 2,4,6-trihydroxybenzaldehyde ($p < 0.05$), green tea polyphenols, geraniol and 2-hydroxy-5-methoxybenzaldehyde ($p < 0.01$) all showed a significant decrease in the collapse pressure. When comparing the effect caused by these compounds, there was no significant difference ($p > 0.05$) between them. For DOPE monolayer, none of the compounds tested showed any significant effect ($p > 0.05$) on the collapse pressure. For DPPG monolayer, only geraniol showed a significant decrease ($p < 0.05$) in the collapse pressure but not other compounds. For DOPG monolayer, the result was similar to the DPPG monolayer, only geraniol showed a significant decrease ($p < 0.05$) in the collapse pressure but not other compounds. For CL monolayer, 2,5-dihydroxybenzaldehyde and geraniol had caused a significant decrease ($p < 0.05$ for 2,5-dihydroxybenzaldehyde and $p < 0.001$ for geraniol) in the collapse pressure, while the effect caused by geraniol was significantly greater ($p < 0.05$) than 2,5-dihydroxybenzaldehyde.

Table 6.3 The values of collapse pressure (mN/m) of lipid monolayers DPPE, DOPE, DPPG, DOPG and CL compressed on subphases containing pure water with and without naturally occurring antimicrobial compounds.

	DPPE	DOPE	DPPG	DOPG	CL
Water	**55**	**38**	**49**	**40**	**49**
2,5-Dihydroxy benzaldehyde	47 No significant effect ($p > 0.05$)	39 No significant effect ($p > 0.05$)	50 No significant effect ($p > 0.05$)	41 No significant effect ($p > 0.05$)	40 Significantly decreased ($p < 0.05$)
2,4,6-Trihydroxy benzaldehyde	44 Significantly decreased ($p < 0.05$)	44 No significant effect ($p > 0.05$)	54 No significant effect ($p > 0.05$)	45 No significant effect ($p > 0.05$)	47 No significant effect ($p > 0.05$)
Apple E polyphenols	55 No significant effect ($p > 0.05$)	41 No significant effect ($p > 0.05$)	54 No significant effect ($p > 0.05$)	42 No significant effect ($p > 0.05$)	48 No significant effect ($p > 0.05$)
Green tea polyphenols	38 Significantly decreased ($p < 0.01$)	37 No significant effect ($p > 0.05$)	54 No significant effect ($p > 0.05$)	44 No significant effect ($p > 0.05$)	45 No significant effect ($p > 0.05$)
***trans*-Cinnamaldehyde**	45 No significant effect ($p > 0.05$)	34 No significant effect ($p > 0.05$)	44 No significant effect ($p > 0.05$)	42 No significant effect ($p > 0.05$)	50 No significant effect ($p > 0.05$)
Carvacrol	45 No significant effect ($p > 0.05$)	31 No significant effect ($p > 0.05$)	42 No significant effect ($p > 0.05$)	36 No significant effect ($p > 0.05$)	45 No significant effect ($p > 0.05$)
Geraniol	41 Significantly decreased ($p < 0.01$)	30 No significant effect ($p > 0.05$)	38 Significantly decreased ($p < 0.05$)	31 Significantly decreased ($p < 0.05$)	31 Significantly decreased ($p < 0.001$)
2-Hydroxy-5-methoxy benzaldehyde	39 Significantly decreased ($p < 0.01$)	39 No significant effect ($p > 0.05$)	41 No significant effect ($p > 0.05$)	39 No significant effect ($p > 0.05$)	41 No significant effect ($p > 0.05$)

Table 6.4 shows the calculated maximal values of compression modulus of different monolayers, on subphase containing pure water with and without naturally occurring antibacterial compound. The compression modulus could be served as an indicator of membrane fluidity, the lower the value, the higher the fluidity of the monolayer.

For DPPE monolayer, all of the compounds tested had shown a significant decrease ($p < 0.001$) in the maximal values of compression modulus, which indicated that all of the compounds could fluidize the membrane. The fluidizing effect caused by green tea polyphenols, carvacrol and geraniol was similar, which was significantly greater ($p < 0.05$) than the fluidizing effect caused by 2,5-dihydroxybenzaldehyde, 2,4,6-trihydroxybenzaldehyde, apple E polyphenols, *trans*-cinnalaldehyde and 2-hydroxy-5-methoxybenzaldehyde.

For DOPE monolayer, all of the compounds tested showed a significant decrease ($p < 0.001$ to 0.05) in the maximal values of compression modulus. With the exception of 2,4,6-trihydroxybenzaldehyde, which showed a significant increased ($p < 0.05$) in the maximal values of compression modulus. Carvacrol had the highest fluidizing effect in DOPE monolayer, followed by geraniol, then *trans*-cinnalaldehyde and 2-hydroxy-5-methoxybenzaldehyde.

For DPPG monolayer, all of the compounds tested showed a significant decrease ($p < 0.001$) in the maximal values of compression modulus, which indicated that all of the compounds could fluidize the membrane. The fluidizing effect caused by carvacrol was significantly higher ($p < 0.001$) than all other compounds, while apple E polyphenols had shown the least fluidizing effect.

For DOPG monolayer, 2,4,6-trihydroxybenzaldehyde caused a significant increase ($p < 0.01$) in the maximal values of compression modulus; while apple E polyphenols and green tea polyphenols had no significant effect ($p > 0.05$); and the remaining compounds showed a significant decreased ($p < 0.01$ to 0.001). The fluidizing effect caused by geraniol was the highest, while 2,5-dihydroxybenzaldehyde was the lowest.

For CL monolayer, all of the compounds tested caused a significant decrease ($p < 0.05$ to <0.001) in the maximal values of compression modulus, which indicated that all of the compounds could fluidize the membrane. The fluidizing effect caused by geraniol was the highest, which was the same as for DOPG monolayer.

Table 6.4 The maximal values of compression modulus (mN/m) of lipid monolayers DPPE, DOPE, DPPG, DOPG and CL compressed on subphases containing pure water with and without naturally occurring antimicrobial compounds.

	DPPE	DOPE	DPPG	DOPG	CL
Water	**183**	**122**	**256**	**116**	**169**
2,5-Dihydroxy benzaldehyde	109 Significantly decreased ($p < 0.001$)	109 Significantly decreased ($p < 0.01$)	174 Significantly decreased ($p < 0.001$)	97 Significantly decreased ($p < 0.01$)	150 Significantly decreased ($p < 0.01$)
2,4,6-Trihydroxy benzaldehyde	121 Significantly decreased ($p < 0.001$)	137 Significantly increased ($p < 0.05$)	183 Significantly decreased ($p < 0.001$)	135 Significantly increased ($p < 0.01$)	151 Significantly decreased ($p < 0.05$)
Apple E polyphenols	96 Significantly decreased ($p < 0.001$)	109 Significantly decreased ($p < 0.05$)	194 Significantly decreased ($p < 0.001$)	111 No significant effect ($p > 0.05$)	149 Significantly decreased ($p < 0.01$)
Green tea polyphenols	84 Significantly decreased ($p < 0.001$)	98 Significantly decreased ($p < 0.001$)	172 Significantly decreased ($p < 0.001$)	115 No significant effect ($p > 0.05$)	147 Significantly decreased ($p < 0.01$)
***trans*-Cinnamaldehyde**	99 Significantly decreased ($p < 0.001$)	80 Significantly decreased ($p < 0.001$)	172 Significantly decreased ($p < 0.001$)	80 Significantly decreased ($p < 0.001$)	120 Significantly decreased ($p < 0.001$)
Carvacrol	75 Significantly decreased ($p < 0.001$)	52 Significantly decreased ($p < 0.001$)	96 Significantly decreased ($p < 0.001$)	60 Significantly decreased ($p < 0.001$)	86 Significantly decreased ($p < 0.001$)
Geraniol	87 Significantly decreased ($p < 0.001$)	65 Significantly decreased ($p < 0.001$)	142 Significantly decreased ($p < 0.001$)	42 Significantly decreased ($p < 0.001$)	75 Significantly decreased ($p < 0.001$)
2-Hydroxy-5-methoxy benzaldehyde	105 Significantly decreased ($p < 0.001$)	80 Significantly decreased ($p < 0.001$)	177 Significantly decreased ($p < 0.001$)	87 Significantly decreased ($p < 0.001$)	116 Significantly decreased ($p < 0.001$)

Table 6.5 shows the recorded surface potential of different monolayers at their condensed state, on subphase containing pure water with and without naturally occurring antibacterial compound. The surface potential represents the total dipole moment in the monolayer system.

For DPPE monolayer, compounds 2,5-dihydroxybenzaldehyde, apple E polyphenols, green tea polyphenols, *trans*-cinnalaldehyde and geraniol significantly decreased ($p < 0.01$) the surface potential at the condensed state, which indicated a decrease in total dipole moment in the DPPE monolayer system. Comparing the effect caused by these compounds, it was not significantly different ($p > 0.05$). Compounds 2-hydroxy-5-methoxybenzaldehyde and carvacrol did not cause significant effect on the total dipole moment in the DPPE monolayer system ($p > 0.05$).

For DOPE monolayer, compounds *trans*-cinnalaldehyde and geraniol significantly decreased ($p < 0.001$) the surface potential at the condensed state to a similar extent; compounds 2,5-dihydroxybenzaldehyde, apple E polyphenols and green tea polyphenols did not cause significant effect on the total dipole moment ($p > 0.05$); while compounds 2-hydroxy-5-methoxybenzaldehyde and carvacrol significantly increased ($p < 0.001$) the total dipole moment in the DOPE monolayer system and the effect caused by 2-hydroxy-5-methoxybenzaldehyde was significantly larger ($p < 0.001$).

For DPPG monolayer, compounds apple E polyphenols and 2-hydroxy-5-methoxybenzaldehyde did not cause significant effect on the total dipole moment ($p > 0.05$); all other compounds significantly decreased ($p < 0.001$ to 0.05) the surface potential at the condensed state, while the level of decrease between these compounds was significantly different ($p < 0.05$) in the order of *trans*-cinnalaldehyde and geraniol > 2,5-dihydroxybenzaldehyde and carvacrol > green tea polyphenols.

For DOPG monolayer, compounds 2,5-dihydroxybenzaldehyde and apple E polyphenols did not cause significant effect on the total dipole moment ($p > 0.05$); compound 2-hydroxy-5-methoxybenzaldehyde significantly increased ($p < 0.001$) the surface

potential at the condensed state and green tea polyphenols caused a slight but yet significantly increase ($p < 0.05$) in the surface potential; while compounds *trans*-cinnalaldehyde, carvacrol and geraniol significantly decreased ($p < 0.001$) the surface potential, the effect of geraniol > *trans*-cinnalaldehyde > carvacrol.

For CL monolayer, compound carvacrol did not cause significant effect on the total dipole moment ($p > 0.05$); compounds 2,5-dihydroxybenzaldehyde, apple E polyphenols, green tea polyphenols and 2-hydroxy-5-methoxybenzaldehyde significantly increased ($p < 0.001$ to 0.05) the surface potential at the condensed state to a similar extent; while compounds *trans*-cinnalaldehyde and geraniol significantly decreased ($p < 0.001$) the surface potential, the effect of geraniol was significantly larger ($p < 0.01$) than *trans*-cinnalaldehyde.

For monolayer of total lipids of *Map*, compound 2-hydroxy-5-methoxybenzaldehyde did not cause significant effect on the total dipole moment ($p > 0.05$); compounds 2,5-dihydroxybenzaldehyde, *trans*-cinnalaldehyde, carvacrol and geraniol significantly decreased ($p < 0.001$) the surface potential to different level, in the order of geraniol > *trans*-cinnalaldehyde > carvacrol > 2,5-dihydroxybenzaldehyde.

Table 6.5 The reading of surface potential (mV) of lipid monolayers DPPE, DOPE, DPPG, DOPG, CL and *Map* total lipids at condensed state, compressed on subphases containing pure water with and without naturally occurring antimicrobial compounds.

	DPPE	**DOPE**	**DPPG**	**DOPG**	**CL**	***Map* total lipids**
Water	**591**	**181**	**424**	**162**	**258**	**365**
2,5-Dihydroxy benzaldehyde	278 Significantly decrease ($p < 0.001$)	160 No significant effect ($p > 0.05$)	224 Significantly decreased ($p < 0.001$)	156 No significant effect ($p > 0.05$)	434 Significantly increased ($p < 0.01$)	221 Significantly decrease ($p < 0.001$)
Apple E polyphenols	315 Significantly decreased ($p < 0.001$)	210 No significant effect ($p > 0.05$)	454 No significant effect ($p > 0.05$)	191 No significant effect ($p > 0.05$)	391 Significantly increased ($p < 0.01$)	nt[a]
Green tea polyphenols	335 Significantly decreased ($p < 0.01$)	187 No significant effect ($p > 0.05$)	370 Significantly decreased ($p < 0.05$)	198 Significantly increased ($p < 0.05$)	380 Significantly increased ($p < 0.05$)	nt
***trans*-Cinnamaldehyde**	292 Significantly decreased ($p < 0.001$)	69 Significantly decreased ($p < 0.001$)	64 Significantly decreased ($p < 0.001$)	-67 Significantly decreased ($p < 0.001$)	78 Significantly decreased ($p < 0.001$)	30 Significantly decrease ($p < 0.001$)
Carvacrol	526 No significant effect ($p > 0.05$)	272 Significantly increased ($p < 0.001$)	182 Significantly decreased ($p < 0.001$)	54 Significantly decreased ($p < 0.001$)	281 No significant effect ($p > 0.05$)	181 Significantly decrease ($p < 0.001$)
Geraniol	298 Significantly decreased ($p < 0.001$)	74 Significantly decreased ($p < 0.001$)	20 Significantly decreased ($p < 0.001$)	-200 Significantly decreased ($p < 0.001$)	-95 Significantly decreased ($p < 0.001$)	-41 Significantly decrease ($p < 0.001$)
2-Hydroxy-5-methoxy benzaldehyde	596 No significant effect ($p > 0.05$)	379 Significantly increased ($p < 0.001$)	445 No significant effect ($p > 0.05$)	278 Significantly increased ($p < 0.001$)	452 Significantly increased ($p < 0.001$)	366 No significant effect ($p > 0.05$)

[a] The naturally occurring antibacterial compound was not tested against that lipid.

6.4 Discussion

6.4.1 Surface pressure

Lipid monolayers were formed at the air-liquid interface to mimic the surface of the bacterial membrane by using DPPE, DPPG, DOPE, DOPG and cardiolipin. Considering the surface pressure-area isotherms of DPPE, the naturally occurring antimicrobial compounds caused different degrees of changes, either increase or decrease, in the lift-off value and the slope of the isotherms. Despite the change of lift-off value, either increased or decreased; the compression modulus might not be affected. The factor that determines compressibility of the isotherm is its slope.

In general, natural compounds caused an increase in lift-off values on lipid monolayers, with the exception of three compounds, 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde and apple E polyphenols, which caused a decrease in the lift-off values on DPPE monolayer and 2,5-dihydroxybenzaldehyde on DPPG and DOPG, 2,4,6-trihydroxybenzaldehyde on DPPG, DOPE and DOPG and 2-hydroxy-5-methoxybenzaldehyde on DPPE, which showed no siginificant effect ($p > 0.05$). The increase in the lift-off value indicated these antimicrobial compounds could be incorporated into the lipid monolayers and/or they can modify the structure of lipid matrix and exert their bactericidal action, these changes might be due to electrostatic interactions between lipids, water molecules and antimicrobial compounds. An example of antimicrobial substance disrupting the lipid monolayer is shown in figure 6.15.

As the three compounds 2,4,6-trihydroxybenzaldehyde, 2,5-dihydroxybenzaldehyde and apple E polyphenols caused a decrease in the lift-off values, which indicated they could induce better packing effectiveness of DPPE lipid monolayer, this may suggest they have a different mode of interaction on DPPE molecules.

6.4.2 Compressibility

This biophysical experiment indicated that antimicrobial compounds interact differently with the lipid rafts in different lipids depending on the number of saturations, electrical

charge at the polar head and dipole moment. Antimicrobial molecules were found to interact more easily with the gas or liquid phase of the monolayer than solid phase. This observation was in agreement with the literature reports that lipid rafts are characterized by compact lipid arrangements. It may be due to the electrostatic interaction between charged head groups and antimicrobials inserted between hydrophobic tails. It has been suggested that shape and size of lipid rafts are determined by competition between the line tension at the raft boundary and the electrostatic repulsion between molecular polar heads (Diociaiuti et al., 2004).

The changes of compressibility modulus reflect the physical state of the lipid monolayer compressed on the surface on different subphases. This can be calculated as a function of surface pressure π with pure water and natural compounds from compression isotherms. The higher the value of compressibility modulus, the higher the rigidity of the model membrane and vice versa a low value of compressibility modulus indicates a high fluidity of the model membrane (Sabatini et al., 2008).

Only one compound 2,4,6-trihydroxybenzaldehyde caused a slight rigidifying effect on both DOPE and DOPG and apple E polyphenols and green tea polyphenols showed no significant effect ($p > 0.05$) on DOPG. All other compounds exhibited a fluidizing effect, either small or large. Oil compounds showed a greater fluidizing effect compared to the powder compounds, especially carvacrol and geraniol, as they showed the highest fluidizing effect in all the monolayer tested.

Results of this study indicated that natural antimicrobial compounds generally modify the lipid monolayer structure by incorporating into the lipid monolayer, generating aggregation/raft of antimicrobials and lipids and increasing the membrane fluidity. This change of fluidity may affect the dipole density, the higher the fluidity of the monolayer, the lower the dipole density. The thickness of the membrane (figure 6.14) could also be affected by antimicrobials, by modifying the orientation of lipid molecules/rafts to the water surface more or less perpendicular or parallel (figure 6.12 and 6.16).

6.4.3 Surface potential

According to Dynarowicz-Łątka et al.'s review (2001), the surface potential arised from the total dipole moment of a monolayer system from three components, the dipole moment at the hydrophobic region, the polar region of the lipid molecules, and the aqueous subphase adjacent to the lipid molecules. It is not possible to measure any one of the above parameters individually but the total surface dipole potential of the monolayer.

Our results suggest that natural antimicrobial compounds might change the total dipole moment of the lipid monolayer by modifying the three components together or separately, i.e. the modification of lipid hydration of the monolayer through the arrangement of water molecules just below the polar heads; or modification of the Van der Waals interaction at the hydrophobic region between the hydrocarbon chains, which may reduce the packing effectiveness of the lipid molecules in the monolayer.

When considering the π–A isotherms obtained from the five lipid monolayers compressed on subphase containing pure water only, it was found that DPPE had the smallest lift-off value, followed by DPPG, cardiolipin, DOPE and finally DOPG. It may be due to the highest packing density of DPPE, as DPPE has a zwitterionic polar head and lacks of *cis* double bonds in the fatty acyl chains. DPPG and cardiolipin had a higher lift-off value than DPPE, because of their lack of *cis* double bonds in the fatty acyl chains but both have anionic polar head; the electrostatic repulsion would not allow DPPG and cardiolipin to pack as tight as DPPE. Both DOPE and DOPG exhibited the highest lift-off values because of the presence of two *cis* double bonds in their fatty acyl chains. The *cis* double bond poses a kink in each fatty acyl chain and an increase area per molecule in two-dimensional monolayer system. This area per molecule determines the dipole density of the monolayer. This explains why DPPE not only had the highest packing density but also the highest surface potential.

When considering the surface potential of monolayers in the presence of natural antimicrobial compounds, both an increase and a decrease of surface potential were

observed. In the presence of 2-hydroxy-5-methoxybenzaldehyde, the surface potential of monolayers was either increased or remained unchanged. The powdered compounds also showed slight, or no significant effect ($p > 0.05$) on surface potential of DOPE and DOPG, which were both phospholipids containing two *cis* double bond at their fatty acyl chains. The oil compounds cinnamaldehyde and geraniol showed the biggest decrease in surface potential in all monolayer tested.

Interestingly, cinnamaldehyde showed the strongest antimicrobial effect on *Map* (24 μg/ml), while geraniol did not show any inhibitory effect on *Map* at all and only moderate effect on both *Cl. sporogenes* and *E. coli*. However, geraniol was still able to decrease the surface potential to a similar extent as cinnamaldehyde. Both compounds showed extremely significant effects ($p < 0.001$) on the surface potential of the monolayer of *Map* total lipids, which suggested they had high affinity to the *Map* lipid molecules and they had high ability to modify the arrangement of *Map* lipid molecules and the water molecules adjacent to the lipids. However, this data obtained from the surface potential measurement was not in good agreement with that of the antimicrobial assay, which may imply there could be another mode of action possessed by cinnamaldehyde, apart from disrupting the lipid organization at the membrane.

In addition, as described in the results obtained from the DPPE surface potential measurement, though the trend and values of surface potential at condensed state were similar in the presence of cinnamaldehyde and geraniol, the way of the reorganization of the lipid structure might be different. The living *Map* cultures might be able to repair the disruption in membrane lipid molecules caused by geraniol but not by cinnamaldehyde. On the other hand, anti-*Map* natural compound, 2-hydroxy-5-methoxybenzaldehyde did not cause significant change ($p > 0.05$) of surface potential of monolayer of total *Map* lipids, which may also imply another mode of antimicrobial action.

Recent research has involved the use of monolayer technique to study the mode of action of antimicrobial peptides, which are mainly cationic, based on the electrostatic interaction. It has been found that antimicrobial peptides are especially attracted to

anionic lipids and attached to their phospho-head group, causing reorganization of membrane lipid molecules and membrane disruption. The essential oil components are not well known for their electrostatic charges. However, because they are hydrocarbons having high hydrophobicity property, they are able to partition into the hydrophobic region (fatty acyl chains) of the membrane, exerting non specific hydrophobic interaction, through intercalation between the lipid molecules and disrupt the cell membrane.

Natural antimicrobials changed, either decreased or increased the surface potential of the monolayer of DPPE, DPPG, DOPE, DOPG, CL and total *Map* lipids to different extents. On some occasions, negative surface potential readings were observed, mostly in the presence of cinnamaldehyde and geraniol. The changes might be due to the incorporation of antimicrobial compounds into the liquid-expanded phase and/or the liquid-condensed phase and their interaction with the lipid molecules, thus modify the molecular packing and organization of the lipid rafts. The negative surface potential reading may also indicate that the orientation of the dipoles had rotated by 180°. Some of the results of surface potential measurements were not in good agreement of the antimicrobial assay, e.g. geraniol and 2-hydroxy-5-methoxybenzaldehyde, which may suggest there could be some other mode of action exist, apart from disrupting the lipid organization at the membrane.

Addition of antimicrobials having different dipole moment (in terms of orientation) to the monolayer would reduce the total dipole of the monolayer, while addition of antimicrobials having the same dipole moment to the monolayer would increase the total dipole moment and thus surface potential. Apart from these, the monolayer thickness (or hydrophobic thickness) would also affect the dipole moment of the monolayer. Modification of membrane fluidity might be a consequence of change of monolayer thickness.

To conclude, the possible modes of antimicrobial action of naturally occurring compounds were studied using model membrane systems. In the monolayer model

membrane studies, it was found that natural antimicrobial compounds could modify the lipid monolayer structure by incorporating into the lipid monolayer, forming aggregates/rafts of antimicrobials and lipids, reducing the packing effectiveness of the lipid molecules, increasing the membrane fluidity and altering the total dipole moment in the monolayer system. These results suggested that the antimicrobial activity of natural compounds may exhibit their action via change/interaction with the bacterial cell membrane. Once the physical property of a lipid raft is modified, sequential changes on other aspects might be initiated as a consequence. All these changes may suggest similar events could happen to the biological membranes, as the biological membranes are more complex than monolayer system. Once their physical properties are changed, the membrane may lose its vital function. This study confirmed the antimicrobial compounds, especially oil compounds are targeting the cell membrane and in good agreement with the findings in biological approaches. The finding of a moderate compound, geraniol that exerted significant effects on lipid rafts in the monolayer further suggested the possibility of multiple modes of action antimicrobial naturally occurring compounds. By gaining further insight into the interaction between antimicrobials and membrane lipids and identification of characteristics of the most potent compounds, new therapeutic development based on rational design or modification of existing compounds might be possible.

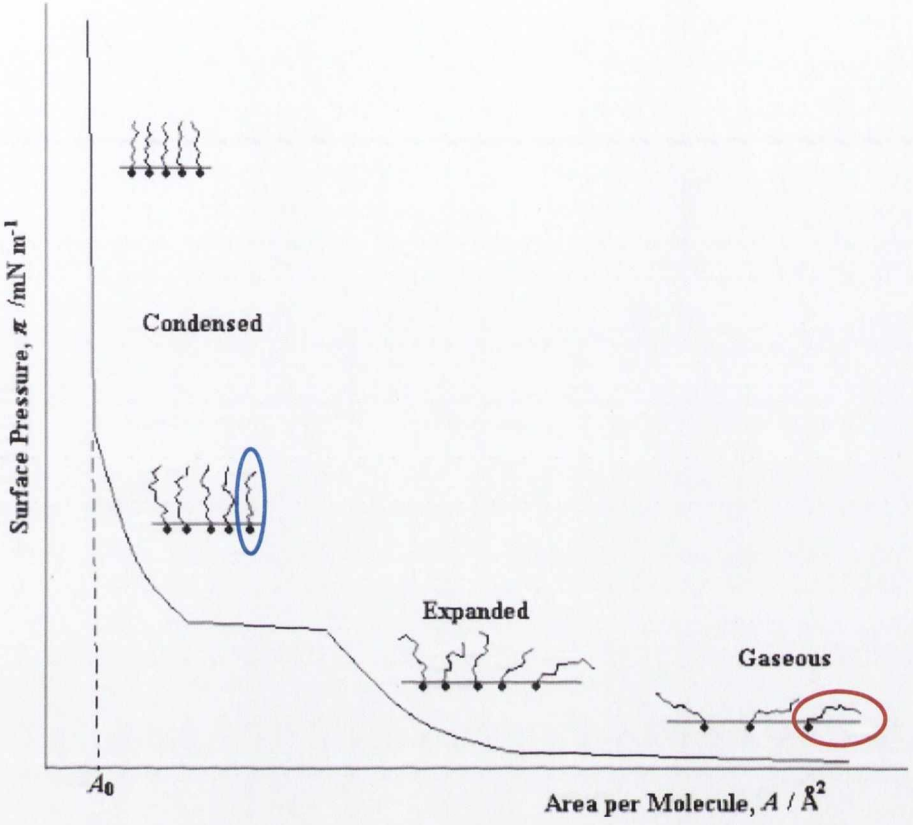

Figure 6.12 A typical π -A isotherm

As shown in figure 6.12, the blue circle indicates a lipid molecule lying perpendicularly to the water subphase, in contrast to the red circle, the lipid molecule is lying almost parallel to the water subphase.

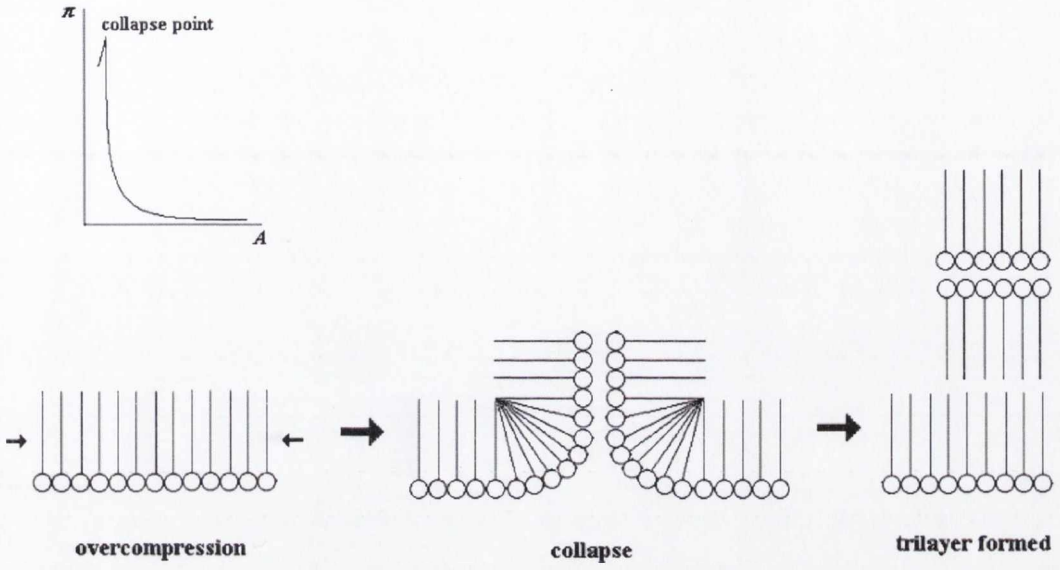

Figure 6.13 Collapse of monolayer upon compression

Figure 6.13 illustrates that upon further compression, the monolayer film can collapse, with film molecules being forced out of the monolayer, resulting in multilayer (often trilayer) structures. The collapse point is shown by a sudden fall in π.

Figures 6.12 and 6.13 were obtained from the online lecture material, by Dr Cooper, S. J., Department of Chemistry, University of Durham.
(http://www.dur.ac.uk/sharon.cooper/lectures/colloids/interfacesweb2.html)

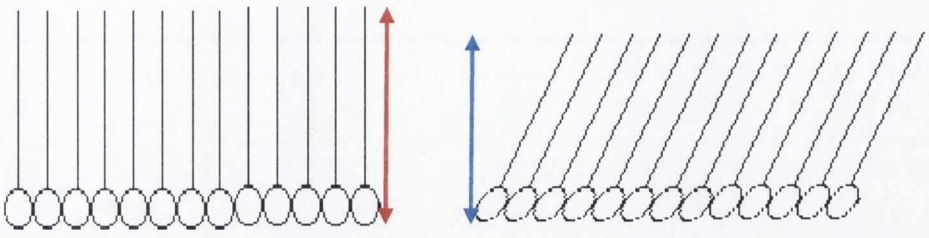

Figure 6.14 Thickness of membrane.

Thickness of membrane could be determined by the angle of lipid molecules form between the water surfaces.

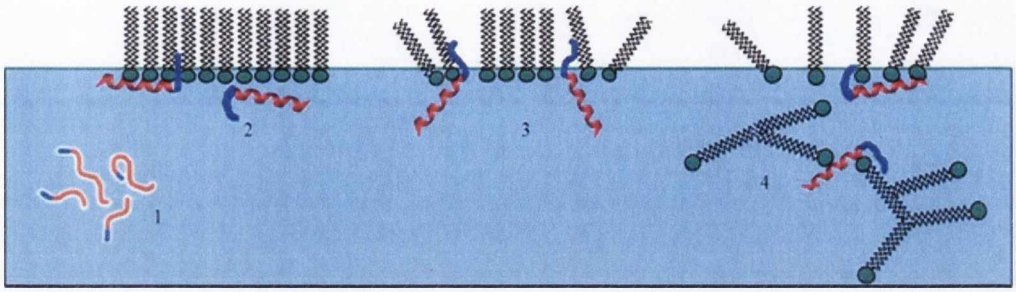

Figure 6.15 Proposed model for antimicrobial peptide/DPPG monolayer interaction

Figure 6.15 illustrates a proposed model for antimicrobial peptide disrupting the lipid monolayer. Pln149a, an antimicrobial peptide produced by lactic acid bacterium, *Lactobacillus plantarum* NRIC 149, presents a random-coil structure in aqueous solution (1), but it is electrostatically attracted to a negative surface (like DPPG), inducing an amphipathic α-helix in its structure (2). The surface packing of the monolayer is gradually altered to accommodate more and more Pln149a from the solution (3), resulting in the disruption of the lipid monolayer (4).

Figure 6.15 was obtained from Lopes et al. (2009). Disruption of *Saccharomyces cerevisiae* by Plantaricin 149 and investigation of its mechanism of action with biomembrane model systems. *Biochimica et Biophysica Acta*. 1788: 2252–2258

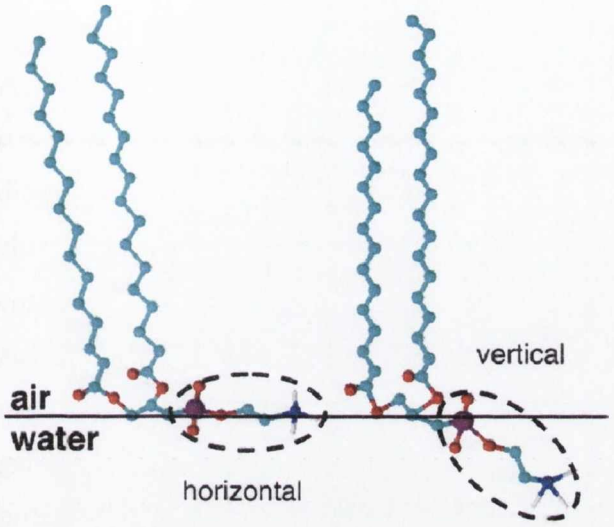

Figure 6.16 Illustration of different orientations of the ethanolamine group at the water/air interface.

Figure 6.16 was obtained from Vila-Romeu et al. (2008). The interaction of cyclodextrins with phosphatidylethanolamine Langmuir monolayers: Influence of the spreading solvent and subphase conditions. *Thin Solid Films*. 516:8852–8859

General Conclusion

Antibiotics were discovered serendipitously to promote the performance of livestock, e.g. decrease mortality rate, prevent disease and enhance growth rate; since this discovery they have been administered routinely in animal husbandry. However, due to changes in consumer preferences such as the demand for natural chemical-free food and concerns regarding the consumption of food containing residues or metabolites of antibiotics, the link between the development of antibiotic resistance and the extensive use of antibiotics in agricultural food production, and the transfer of antibiotic resistant genes from animals to humans via the food chain, the use of antibiotics as growth promoters has been banned in the EU since January 2006. In order to maintain the profit of livestock producers, cope with the growing demand for food, keep the cost of animal food production low and maintain the competitiveness of agricultural foods produced in the EU, there are increasing efforts in searching for natural alternatives to chemical antibiotics.

Such alternatives should be natural, safe, effective and economically viable. Naturally occurring compounds, e.g. plant extracts and essential oils are attracting much attention, as they have been widely used in folk medicine, food seasonings and food preservations since antiquity. They have the potential to protect the host from different infections via natural defence mechanisms; they are also generally recognised as safe and unlikely to cause resistance in pathogens. Many studies have examined their potential use as antimicrobial agents, food preservatives, disease treatments, disease preventers and animal growth enhancers.

This study evaluated the antimicrobial effects of naturally occurring compounds against pathogenic microorganisms relevant to food safety, public health, animal health and welfare. These organisms include *Escherichia coli* (*E. coli*), *Clostridium sporogenes* (*Cl. sporogenes*) and *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). The pathogenic *E. coli* causes rare but severe food borne illness in humans; *Cl. sporogenes* a surrogate for *Cl. perfringens*, causes necrotic enteritis in broiler chickens, affecting their

growth performance and in severe cases resulting in fatality and can also cause food borne illness in humans; *Map* causes Johne's disease in cattle and may play a role in human Crohn's disease.

The overall objectives included determination of the minimum inhibitory concentrations (MICs) of the active compounds, investigation of the possible synergistic effects when combining different natural antimicrobial compounds and investigation of the antimicrobial modes of action of these active compounds. The importance of determining MICs is to identify the potent compounds, which exert antimicrobial activity at a relatively low concentration. The importance of synergistic study is to investigate the possibility of combining the naturally occurring compounds, which would lower the amount used and the cost if these compounds are applicable for use in the food industry. It is important to study the antimicrobial mode of action of these compounds as such understanding could help to modify or aid the rational design of novel therapeutics.

Chapters 2 and Chapter 3 describe the screening of a wide arrange of naturally occurring compounds (30) for their antibacterial activity against *E. coli* K12, *Cl. sporogenes* and *Map*. Seven were found to be active against *E. coli* K12. The most potent compound was 2,5-dihydroxybenzaldehyde, followed by apple E polyphenols, gallic acid, green tea polyphenols, garlic powder, onion powder and oregano oil. Five showed antibacterial activities against *Cl. sporogenes*. The most potent compound was green tea polyphenols, followed by apple E polyphenols, oregano oil, onion oil and carvarcol. Six inhibited the growth of *Map*. The most potent compound was *trans*-cinnamaldehyde, followed by cinnamon oil, carvacrol, oregano oil, 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde. The active compounds are different in chemical structures and can be grouped into benzaldehydes, phenolic compounds, polyphenols, organic acid and essential oils. The results demonstrate that naturally occurring compounds are effective on a wide range of pathogenic bacteria important in terms of food production and human and animal health. The most potent antimicrobial compounds were subjected to mode of action studies.

Currently, there are no drugs approved to effectively treat Johne's disease. The anti-*Map* naturally occurring compounds identified in this study may have therapeutic potential for the treatment of Johne's disease in farm animals, and possibly in human medicine against Crohn's disease. Carvacrol, the major component of the widely consumed oregano oil, and *trans*-cinnamaldehyde, the major constituent of cinnamon oil, are generally regarded as safe (Friedman et al., 2000, Burt, 2004, Adams et al., 2004). However, the information on the safety issues of 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde are still limited. Further research could be carried out on the toxicity testing of these compounds in order to evaluate their potential as additives in animal feedstuffs and human foods.

Chapter 4 explains the study of the possible synergistic effect of a combination of naturally occurring anti-*Map* compounds and amphibian peptides against *Map*, and the combination of naturally occurring compounds and a herbal extract of *Coptis chinensis Franch* (Aw) against *M. smegmatis* MC^2155, a surrogate of *Map*. The amphibian peptides demonstrated no antimicrobial effects against *Map* at the highest concentration tested but lowered the MICs of the naturally occurring compounds 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde in the macrobroth susceptibility testing. This effect, however, was not observed in the microbroth susceptibility testing. The possible reason for such variation might due to a random mutation which occurred during experimentation, the *Map* culture thus became more resistant to the combination of test compounds. Therefore, it is not possible to confirm the existence of a synergistic effect. Nevertheless, when combined naturally at sub-MIC concentrations, the compounds oregano oil and herbal extract Aw display a partial synergistic effect. The impact of synergistic effects on applications can be, to reduce the amount used, the cost, and decrease possible cytotoxicity of antimicrobial natural compounds. The additive effect will also be beneficial as it can reduce the amount of natural compounds used and maintain the sensory acceptance of food products when applied in food preservation technology.

In Chapter 5, the study of cellular responses of animal pathogen *Map* and *E. coli* under the influence of naturally occurring compounds is reported. Cell autolysis was not observed in *Map* and *E. coli* cultures treated with naturally occurring compounds and no detectable leakage of DNA and soluble protein in *Map* cultures was observed. It was found that naturally occurring compounds caused leakage of phosphate ions in *Map* and *E. coli* cultures in relation to time and concentration of the test compounds. However, the leakage of phosphate was not specific to the antimicrobial activity of the compounds, as the leakage of phosphate was also observed in the presence of non-inhibitory compound, vanillic acid, but not in the presence of the negative control (ethanol < 0.6% vol/vol). The leakage of phosphate study indicated that the cell membrane had been weakened by naturally occurring compounds, regardless of their potency. However, the severity of membrane damage is considered low and thought not to affect the cellular function as *Map* and *E. coli* survives in the presence of vanillic acid.

It is generally accepted that naturally occurring compounds target the cell membrane to achieve inhibition due to their hydrophobicity. Once the integrity of the cell membrane is altered by the naturally occurring compounds, the membrane may lose its vital function, sequential changes on other aspects might be initiated as a consequence. For example the membrane may become unstable, which may affect the function of enzymes and proteins attached to the cell membrane and intracellular constituents may leak out, which may affect the intracellular pH, trans-membrane potential and turgor pressure of the cell; such changes may stress the cellular metabolism. On the other hand, a loss of intracellular constituents may also affect the normal function of the enzymes in the cells.

Naturally occurring compounds, cinnamon oil and its constituent cinnamaldehyde had caused a significant decline in intracellular ATP concentration but no leakage of ATP to the extracellular environment was observed. The decline of intracellular ATP concentration might due to cinnamon oil and cinnamaldehyde which cause a more rapid hydrolysis of intracellular ATP or an inhibition of ATP synthesis. Carvacrol and oregano oil caused a decrease in intracellular ATP concentration at the beginning of incubation but the intracellular ATP concentration raised after 2 h of incubation. The initial

decrease of the intracellular ATP concentration might again be due to hydrolysis of intracellular ATP or inhibition of ATP synthesis, while the increase of the intracellular ATP concentration observed later on might due to a "self-defending" mechanism. When considering the final intracellular ATP concentration, it is significantly lower compared to the negative controls. 2,5-dihydroxybenzaldehyde and 2-hydroxy-5-methoxybenzaldehyde did not change the intracellular ATP concentration significantly throughout the incubation period.

Cinnamaldehyde might have caused leakage of cellular constituents or the transformation to a new product in *Map* according to the appearance of a new peak in the absorbance scan of supernatant of *Map* treated with cinnamaldehyde. The above results indicated that naturally occurring compounds may exert different antimicrobial modes of action, though they might be similar in structure and hydrophobicity.

The possible modes of antimicrobial action of naturally occurring compounds were also studied using model membrane systems (Chapter 6). In the monolayer model membrane studies, it was found that natural antimicrobial compounds could modify the lipid monolayer structure by incorporating into the lipid monolayer, forming aggregates/rafts of antimicrobials and lipids, reducing the packing effectiveness of the lipid molecules, increasing the membrane fluidity and altering the total dipole moment in the monolayer system. Oil compounds cinnamaldehyde and genraniol showed the most significant effect. These results suggested that the antimicrobial activity of natural compounds may exhibit their action via change/interaction with the bacterial cell membrane. Once the physical property of a lipid raft is modified, sequential changes on other aspects might be initiated as a consequence. All these changes may suggest similar events could happen to the biological membranes, as the biological membranes are more complex than the monolayer system. Once their physical properties are changed, the membrane may lose its vital function. This study confirmed that the antimicrobial compounds, especially oil compounds, are targeting the cell membrane and in good agreement with the findings in biological approaches. By gaining further insight into the interaction between antimicrobials and membrane lipids and identification of characteristics of the

most potent compounds, new therapeutic developments base on the modification of existing compounds might be possible.

Further studies could be performed *in vivo* in animal models, using the most potent compound, to evaluate the potential of these naturally occurring compounds as feed additives for controlling microbial population in the animal intestines. The antimicrobial growth promoter (AGPs) like effects of the compound candidate for disease prevention and/or growth promotion can also be assessed. Moreover, further studies could be carried out to analyse the relationship between structures, e.g. the number and position of the functional groups and antimicrobial potency of these naturally occurring compounds. Novel compounds could be modified from the existing compounds or rationally designed to exert potent antimicrobial activities. Apart from this, different approaches to mode of action studies (e.g. proteomic studies which analyse the repression and expression of proteins under the influence of antimicrobials; compatible solute studies which analyse the production of substances that could help the cells to withstand stress; and, membrane composition studies which analyse the changes of membrane fatty acid composition in the presence of antimicrobials) might be carried out to gain a deeper understanding of the mechanism of antimicrobial action of each compound. Finally despite the fact that naturally occurring compounds are generally recognized as safe, it is necessary to carry out toxicity tests to establish if the naturally occurring compounds are harmful when consumed by animals or humans.

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